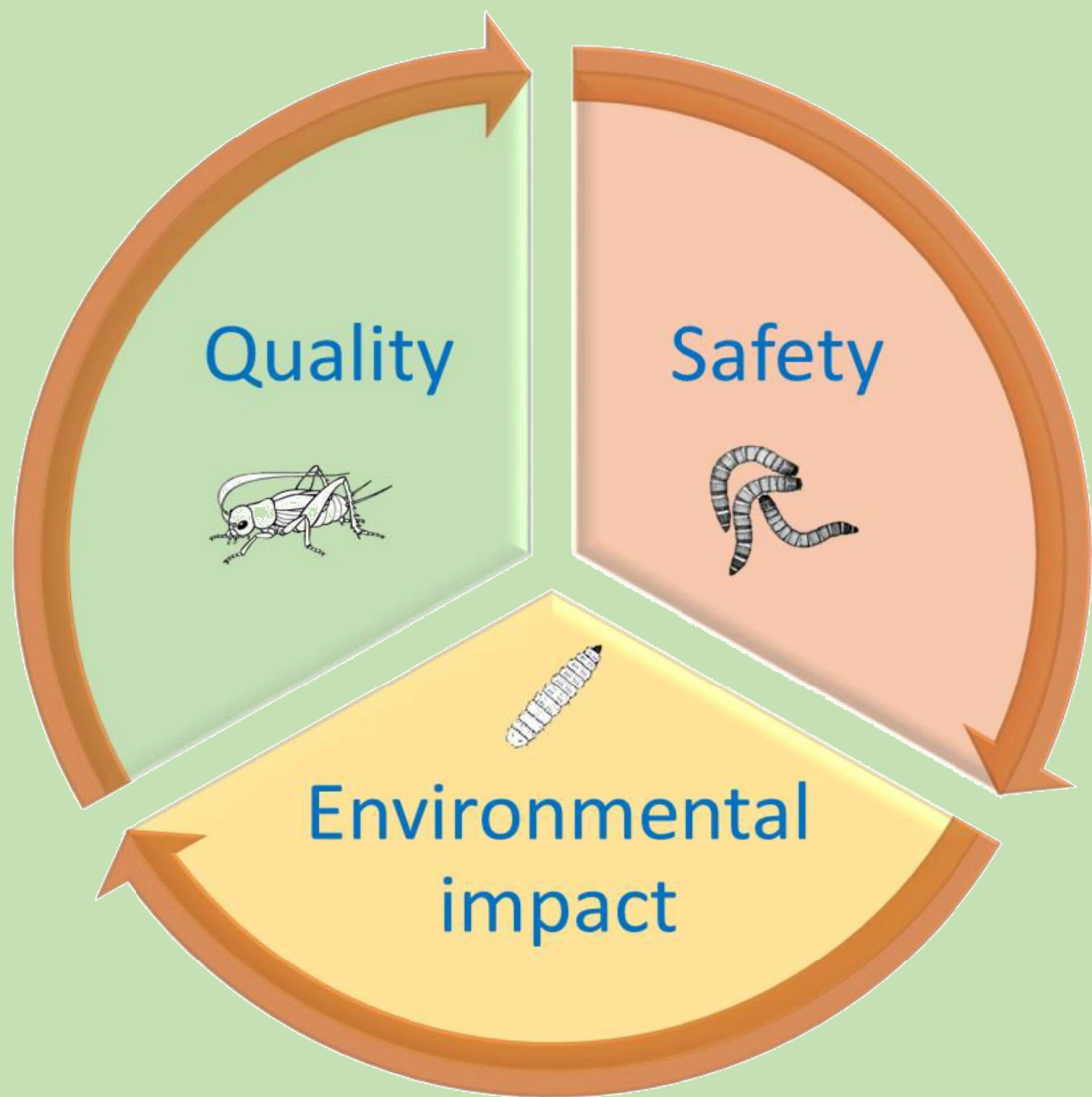


Insect mediated bioconversion in the circular bioeconomy

evaluation of quality, safety and environmental impact



Giacomo Rossi

Insect mediated bioconversion in the circular bioeconomy: *evaluation of quality, safety and environmental impact*



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“I declare that the doctoral thesis here submitted is entirely my own work, written without any unauthorised help by a third party and solely with the assistance referred to in the thesis. I have indicated in the text those text that have been quoted from already published sources, either verbatim or by analogy and all statement based on verbally conveyed information. During the research carried out by me and referred to in the doctoral thesis, I have at all times followed the principles of good scholarly practice as defined in the Status of Justus Liebig University Giessen for Ensuring of Good Academic Practice.”

Potsdam, December 2024

(Giacomo Rossi)

The research work presented in this thesis was carried out in the period November 2019 – October 2024 at the Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) – Potsdam (Germany).

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Summary

Industrially farmed insects are receiving increasing attention within the modern bioeconomy. Due to a diverse range of microorganisms living in their gut, insects are able to grow on biological waste and by-products, recover nutrients and produce new high-quality materials to be exploited within the agriculture, food, feed, medical and industrial sectors. Their high adaptability to different conditions, along with their limited production of greenhouse gasses, low use of resources, high feed conversion and valuable nutritional profile, has also suggested them as potential food and feed sources for the future. However, despite such interesting properties, legislative limitations, often led by fragmented and contradictory knowledge or lack of data, appear to be a serious limitation for their affirmation in the circular bioeconomy.

This thesis focuses on the evaluation of quality, safety and environmental impact of edible insects produced by applying circular economy concepts. **Chapter 1** defines the overall background knowledge motivating the performed experiments. It offers an overview of the research gaps that form the basis for the experimental work and describes the overall structure of the thesis. A general introduction covering several aspects concerning the use of insects in the circular bioeconomy, with a specific focus on insects as waste management tool and as future food and feed is summarised in **Chapter 2**.

Chapter 3 represents the core of the thesis. Six experiments, carried out with the aim of answering the research questions defined in Chapter 1 and addressing the identified research gaps are presented and discussed. Specifically, **section 3.1** focuses on the development of a new, non-destructive optical system for the rapid monitoring of quality changes of edible insect products. Fluorescence spectra of dry insect powders, produced by milling insects of five different species belonging to the Orthoptera order, were recorded. The 3D data were organised into an Excitation Emission Matrix (EEM) and analysed through machine learning tools. Five independent fluorescence peaks, each resulting from a different class of chemical compounds, were identified. The obtained results were therefore further applied to detect the oxidation status of insect paste subjected to high hydrostatic pressure (HHP) processing (**section 3.2**). Two experiments were conducted by applying HHP to insect paste at 600 MPa for 5 min. In the first experiment (experiment 2.1), three modified atmosphere packaging (MAP) and normal air packaging were applied after HHP treatment and microbial load as well as lipid and protein oxidation status were monitored during 28 days of refrigerated storage. Results suggested that the combination of HHP with oxygen-free MAP allowed shelf-life extension up to 10 days when refrigeration was applied. However, HHP treatment was found to be responsible for oxidation initialisation. Therefore, the second experiment (experiment 2.2) aimed to validate such observation and test whether addition of commercial antioxidant mixtures before applying HHP could delay the oxidation process. Results showed that although antioxidants exhibited their activity differently during the storage (with the synthetic antioxidant being more active in the initial stage of the storage while the natural antioxidant being more active in a later stage), HHP alone was not responsible for any significant oxidation.

Considering the most interesting aspect of edible insects regarding waste conversion, **section 3.3** investigates the possibility of using black soldier fly larvae (BSFL) as a tool for managing waste from aquaculture production (ASW). Two experiments, the first using fresh (daily-collected) ASW and the second applying anaerobically digested (bulk-accumulated) ASW, were therefore carried out. The first experiment (experiment 3.1) demonstrated that BSFL can conveniently convert ASW; however, the high water content of the initial material required the inclusion of other ingredients in the diet. Mixtures consisting of 75% ASW and 25% of chicken feed were found to be optimal for supporting larval growth and waste conversion. However, the amount of nutrients assimilated by the larvae was extremely low, indicating a significant loss of nutrients. High nutrients retention and consequently a low environmental impact was observed in substrate 100ASW, which was characterised by low dry matter content, resulting in feed limitation for the BSFL. These results were further confirmed in the second experiment (experiment 3.2), which showed that conditions leading to high larval growth performances were negatively correlated with the reduction of environmental impact. Furthermore, experiment 3.2 also displayed low growth ability of BSFL on bulk accumulated ASW, indicating that

fresh ASW should be preferred for insect-mediated aquaculture waste conversion processes. However, although these two experiments showed low nutrient retention by BSFL reared under optimal conditions, the actual amounts of nutrients lost by the larvae in form of gas were not quantified. Therefore, in order to accurately quantify the gas emissions from edible insects during the rearing process, a new open dynamic gas emission chamber was designed and validated for monitoring the greenhouse gas (GHG) emissions from edible insects on a small scale (**section 3.4**). Validation experiment was carried out by rearing BSFL on moistened chicken feed. Obtained results showed that BSFL produced neither CH₄ nor N₂O, while CO₂ production strictly depended on insect growth and metabolic activity. Accordingly, CO₂ emission curves closely resembled the larval growth curves, indicating that an accurate estimation of GHG emission from insects should consider the overall life cycle of the insect, from eggs to harvesting stage, including the neonate stage. Based on the obtained results, an overall conclusion highlighting the main findings and addressing the research questions defined at the beginning of the thesis is presented in the final chapter (**Chapter 4**). Relevance of the overall thesis within the circular bioeconomy framework and the future steps needed to promote a full transition to the circular bioeconomy, are also illustrated in this chapter.

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List of abbreviations

Abbreviation	Description
AAFCo	Association of American Feed Control
AM	Total aerobic mesophilic bacteria
AMN	Total anaerobic mesophilic bacteria
AMPs	Antimicrobial peptides
ASW	Aquaculture solid waste
AV	Acid value
BSFL	Black soldier fly larvae
CFIA	Canadian Food Inspection Agency
CO ₂ -eq	CO ₂ equivalent
EEM	Excitation emission matrix
GHG	Greenhouse gas
GMP	Good manufacturing practices
GRAS	Generally recognised as safe
GWP	Global warming potential
HHP	High hydrostatic pressure
L:D	Light:Dark
MAP	Modified atmosphere packaging
MS	Aerobic mesophilic bacteria spore
PARAFAC	Parallel factor analysis
POV	Peroxide value
RAS	Recirculating aquaculture system
RH	Relative humidity
TBARs	Thiobarbituric acid substances
Y&M	Yeasts and Moulds
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength

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1. Motivation and structure of the thesis

1.1 Background

A recent estimation carried out by the United Nation predicted that the world's population, which is expected to reach 8.2 billion people by the end of 2024, will keep increasing up to 10.3 billion people by the 2085 (U.N., 2024). Such a scenario, together with the rising of average income and population welfare, will lead to an unavoidable increase in food demand, with 56% more food needed for ensuring food security by 2050 (Searchinger *et al.*, 2019; van Dijk *et al.*, 2021).

Currently, approximately 4 billion metric tonnes of food are produced every year (Kummo *et al.*, 2012). Although such an amount of food appears to be sufficient to feed the current world population (Wilmoth *et al.*, 2023), unequal distribution of the resources, together with insecurity and political, economic and climatic crises, has led to contradictory situations. Approximately 10% of the world population (828 million people) is experiencing hunger on daily bases (FAO, 2022), while roughly 38% of the population is suffering overweight and obesity (Koliaki *et al.*, 2023). Additionally, roughly one-third (1.3 billion tonnes) of the food is lost or wasted every year (Ojha *et al.*, 2020), further exacerbating the food security problem. Therefore, it is clear that significant social changes aiming to mitigate inequalities and ensure a fairer distribution of available resources are needed.

On the other hand, a significant increase in the food production is required. Previous studies have suggested that to encounter the global food demand, crop production should at least double by 2050, compared to the data of early 2000 (Tilman *et al.*, 2011). Increasing food production will result in a higher environmental impact, with biodiversity loss, nutrients loss, soil erosion pollution and anthropogenic greenhouse gas (GHG) emissions expected to increase significantly (Godfray *et al.*, 2010). The current food system already exhibits a high environmental footprint, accounting for approximately 50% of habitable land use, 70% of the available freshwater consumption and 23-34% of the global anthropogenic GHG emissions (Halpern *et al.*, 2022). Further intensification of agriculture without introducing substantial changes is unfeasible. Therefore, alternative possible solutions have been proposed to reduce the environmental impact of the food system while meeting the need for increased food production. Among these, several strategies including reduction of meat consumption, promotion of plant-based diets, reduction of food waste and application of technological and managerial improvements have shown interesting results in the short time (McGreevy *et al.*, 2022). However, considering a longer period, no actual improvements have been reached, suggesting that a radical transformation in the overall agri-food system from the primary production to the final consumption and a reconceptualisation of human metabolism, food practices and lifestyles, are needed (Poore and Nemecek, 2018). Adoption of novel foods has been suggested as an interesting tool for reaching such transformations (Mazac *et al.*, 2022; Parodi *et al.*, 2018). Among the novel foods, edible insects have shown high ability to perfectly combine environmental impact reduction with the need of increasing the global food production (Lange and Ykiko, 2023).

1.2 Research questions and structure of the work

Although insects have been described as a novel source of biologically active products and environmental services, knowledge related to the quality, safety and environmental impact of edible insects produced in accordance with the circular bioeconomy are still limited. Despite the rapidly growing literature on the use of insects as waste bioconversion tool and as novel food and feed source, most studies aim to address multiple issues simultaneously, often forgetting that different questions may require different approaches. Accordingly, when the waste conversion is the objective, aspects connected to insect biomass production are often prioritised, resulting in processes optimised for insect growth rather than for effective waste conversion and environmental impact reduction. Finding a right compromise between the two objectives may not always be beneficial, especially at a stage

where the legislation remains conservative and does not allow an indiscriminate use of insects grown on waste for food or feed purposes.

On the other hand, when biomass production is the main objective, important aspects of the effective valorisation of the obtained insects are often overlooked, resulting in final products lacking key characteristics that are essential for their success in the market. Consequently, edible insects continue to face significant challenges in successfully entering the food and feed market.

Based on the considerations above, five research gaps were identified:

- (i) Proposed processing methods for edible insects are limited. While they are able to guarantee a safe final product, they often negatively impact nutritional and organoleptic quality, which can affect the consumer perception and overall acceptance.
- (ii) Storage studies carried out on insect-derived products are limited, indicating that little is known on how to guarantee quality and safety of the product over time.
- (iii) Use of waste as insect rearing substrate is a largely accepted strategy for waste reduction, biomass production and circular bioeconomy promotion. However, when waste is used, insect biomass production is usually the target, while waste reduction to reduce their environmental impact is often neglected. Efforts oriented to the definition of the best strategies for reducing the environmental impact of the insect-mediated waste bioconversion process while promoting the waste conversion and ensuring acceptable insect biomass production are therefore required.
- (iv) Side-streams from industry and agriculture frequently studied for raising insects are often seasonal and not standardised. Therefore, the development of protocols for composite diets allowing continuous and elevate growth rates are necessary.
- (v) Insect farming has a lower ecological footprint when compared with other livestock production systems such as cattle, pigs or hens. This fact represents one of the main reasons to promote insects as novel food and feed source. However, data related to the environmental impact of insect rearing are still scarce and imprecise. Defining standard and more accurate systems for measuring the environmental impact of edible insects is needed for better utilisation of their potential within the circular bioeconomy concept.

Based on these research gaps, this thesis aims to:

- (i) Test new processing and storage methods on edible insect products with the aim of increasing sensorial and chemical properties of the insect-based products.
- (ii) Evaluate the ability of insects to growth and convert novel biological agricultural-related side-streams.
- (iii) Define strategies for improving waste bioconversion performances while ensuring nutrients recovery and low environment impact.
- (iv) Develop a new and reliable method for fast estimation of the greenhouse gas (GHG) emissions associated to insect rearing processes.

Accordingly, the thesis is organised in four main chapters encompassing the three main themes of the work: quality, safety and environmental impact (figure 1). **Chapter 1** (current chapter) offers an overview of the main problems of the current economic model and introduces the research questions motivating the present thesis. **Chapter 2** consists of a general introduction, where the importance of edible insects in promoting a transition toward a new circular development model is presented. In the following chapter (**Chapter 3**), the experimental work carried out with the aim of answering the abovementioned research questions is presented and the main outputs are discussed. A general conclusion, summarising the findings and highlighting their relevance within the circular bioeconomy framework is finally offered in **Chapter 4**.

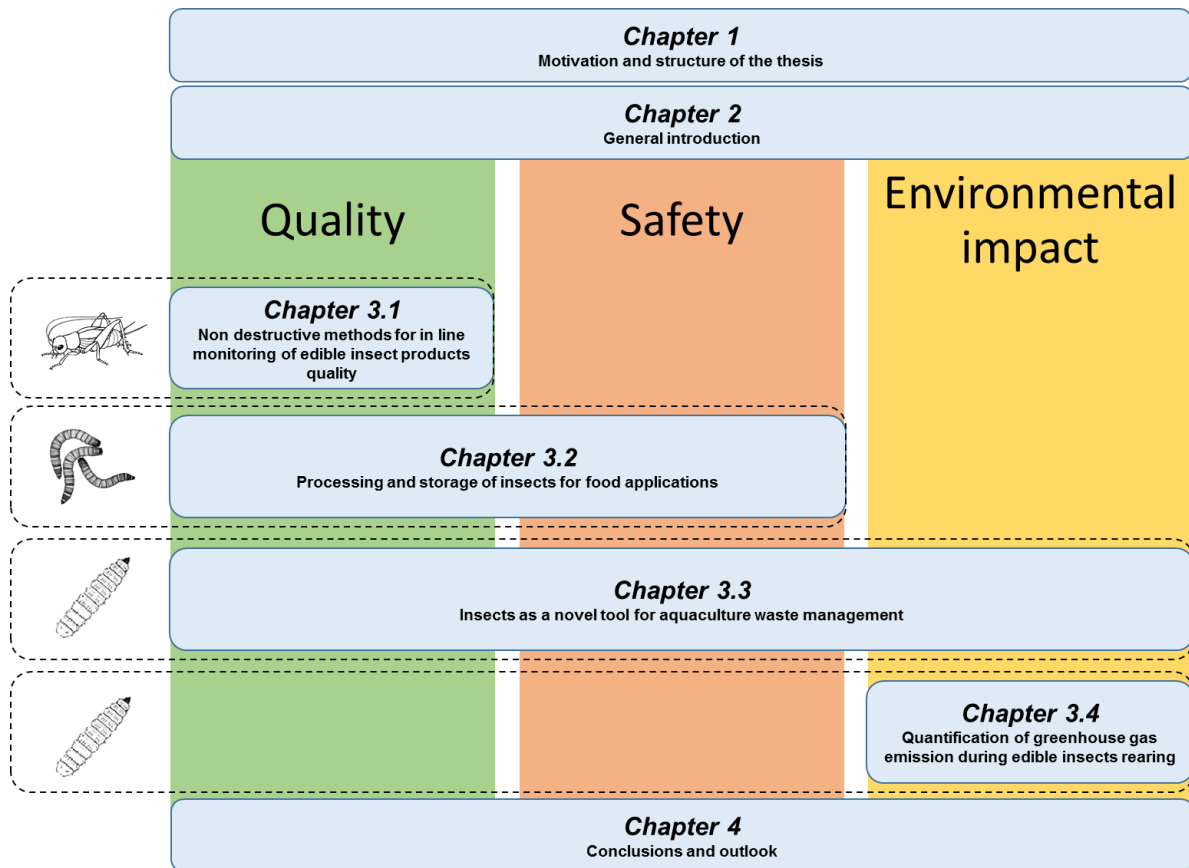


Figure 1. Structure and organisation of the thesis.

These thesis chapters are based on the work published in (or submitted to) peer-reviewed journals and book chapters, which are attached as annex. Information about journal articles and book chapters is summarised in the table 1.

Table 1. List of published/submitted journal articles and book chapters relevant to the thesis and their linkage to the thesis chapters .

No.	Contribution (year)	Title	Authors	Journal [JCR Impact Factor]/Book	Thesis Chapter	Topic(s)
1	Book chapter (2024)	Other applications of insects in the agrifood sector	Rossi. G., Psarianos. M., Schlüter. O.K., Ojha. S.	Insects as Food and Food Ingredients: Technological Improvements Sustainability and Safety Aspects (2024). Pages 215-235. Academic Press. Elsevier. https://doi.org/10.1016/B978-0-323-95594-2.00012-4	Chapter 1	Introduction
2	Book chapter (2023)	Protein from insects—a new biosphere of opportunity	Rossi. G.*, Psarianos. M.*, Schlüter. O.K., Ojha. S.	Future Proteins: Sources. Processing. Applications and the Bioeconomy (2023). Pages 173-194. Academic Press. Elsevier. https://doi.org/10.1016/B978-0-323-91739-1.00009-X	Chapter 1	Introduction
3	Review article (2021)	Edible insect processing pathways and implementation of emerging technologies	Ojha. S., Bußler. S., Psarianos. M., Rossi. G., Schlüter. O.K.	Journal of Insects as Food and Feed. 7(5) – Pages 877-900. https://doi.org/10.3920/JIFF2020.0121 [JCR 2022. IF: 5.7]	Chapter 1; Chapter 3.2	Quality Safety
4	Book chapter (2023)	Storage and packaging of edible insects	Rossi. G., Ojha. S., Pathak. N., Mahajan. P., Schlüter. O.K.	Edible Insects Processing for Food and Feed 1 st Edition (2023). Pages 261-276. CRC Press. Taylor & Francis Group. https://doi.org/10.1201/9781003165729	Chapter 2	Quality Safety

5	Research article (2021)	Fluorescence-based characterisation of selected edible insect species: Excitation emission matrix (EEM) and parallel factor (PARAFAC) analysis	Rossi. G. , Durek. J., Ojha. S., Schlüter. O.K.	Current Research in Food Science. 4 (2021). Pages 862-872. https://doi.org/10.1016/j.crfs.2021.11.004 [JCR 2023. IF: 6.2]	Chapter 3.1	Quality
6	Research article (2023)	Microbial Growth and Physicochemical Quality Changes During Modified Atmosphere Storage of High Hydrostatic Pressure Processed <i>Tenebrio molitor</i> Paste	Ojha. S.*. Rossi. G.*. Pathak. N., Durek. J., Mahajan. P., Schlüter. O.K.	Food and Bioprocess Technology. 17. 1914-1925 (2024). https://doi.org/10.1007/s11947-023-03235-6 [JCR 2023. IF: 5.3]	Chapter 3.2	Quality Safety
7	Research article (submitted)	Effect of high hydrostatic pressure processing on quality of antioxidant-enriched <i>Tenebrio molitor</i> paste	Rossi. G. , Giannini. G., Ojha. S., Buten. C., Pedrazzani. C., Caligiani. A., Tappi. S., Schlüter. O.K.	Submitted to: LWT – Food Science and Technology [JCR 2024. IF: 6.0]	Chapter 3.2	Quality Safety
8	Research article (2023)	Fresh aquaculture sludge management with black soldier fly (<i>Hermetia illucens</i> L.) larvae: investigation on bioconversion performances	Rossi. G. , Ojha. S., Müller-Belecke. A., Schlüter. O.K.	Scientific Reports. 13. 20982 (2023). https://doi.org/10.1038/s41598-023-48061-0 [JCR 2023. IF: 3.8]	Chapter 3.3	Quality Safety Environmental impact
9	Research article (submitted)	Insect-mediated valorisation of anaerobically digested aquaculture waste: bioconversion performances, nutritional composition and microbial safety of black soldier fly larvae	Rossi. G. , Ojha. S., Hankel. J., Schlüter. O.K.	Submitted to: Sustainable Food Technology	Chapter 3.3	Quality Safety
10	Research article (2024)	Estimating the dynamics of greenhouse gas emission during black soldier fly larvae growth under controlled environmental conditions	Rossi. G. , Ojha. S., Berg. W., Herppich. W.B., Schlüter. O.K.	Journal of Cleaner Production, 470. 143226 (2024). https://doi.org/10.1016/j.jclepro.2024.143226 [JCR 2024. IF: 9.7]	Chapter 3.4	Environmental impact

*co-first author

2. General introduction

2.1 Insects as a solution: the yellow biotechnology

Recent estimates suggest that more than 5.5 million of insect species are present on Earth (Stork, 2018). Such as biodiversity makes insects important actors for the world economy.

Most people associate insects to pests, dirty environments and infections (Fugano and Soga, 2021; Hoffmann, 2017). Insects are indeed considered enemies to fight, as they are usually responsible of crops losses, low hygiene and diseases spreading (Lemelin et al., 2017; Saunders and Rader, 2020). On the other hand, it is well known that insects carry out several ecosystem services such as pollination, biological control, organic matter decomposition and pollution bio-indication (Rossi et al., 2024; Noriega et al., 2018; Franco et al., 2022; Saunders and Reder, 2020). Therefore, insects might be seen as important allies for facing several problems, including waste management, climate change, environmental pollution and food security (Bashir et al., 2023; Hamam et al., 2024). Understanding the huge potential of these organisms is crucial for reaching the goal of a more sustainable future.

The term “yellow biotechnology” (a.k.a. insect biotechnology), which has been introduced in 2010, refers to the use of insects and insect-derived products (including organs, cells, molecules as well as symbiotic microorganisms) for specific applications in medicine, plant protection, food, feed and related industries (Vilcinskis, 2015). According to it, insects should be considered as bioreactors able to offer several services and produce several goods useful for humans, animals, plants and the environment. An overview of services and products associated to insects is presented in figure 2.

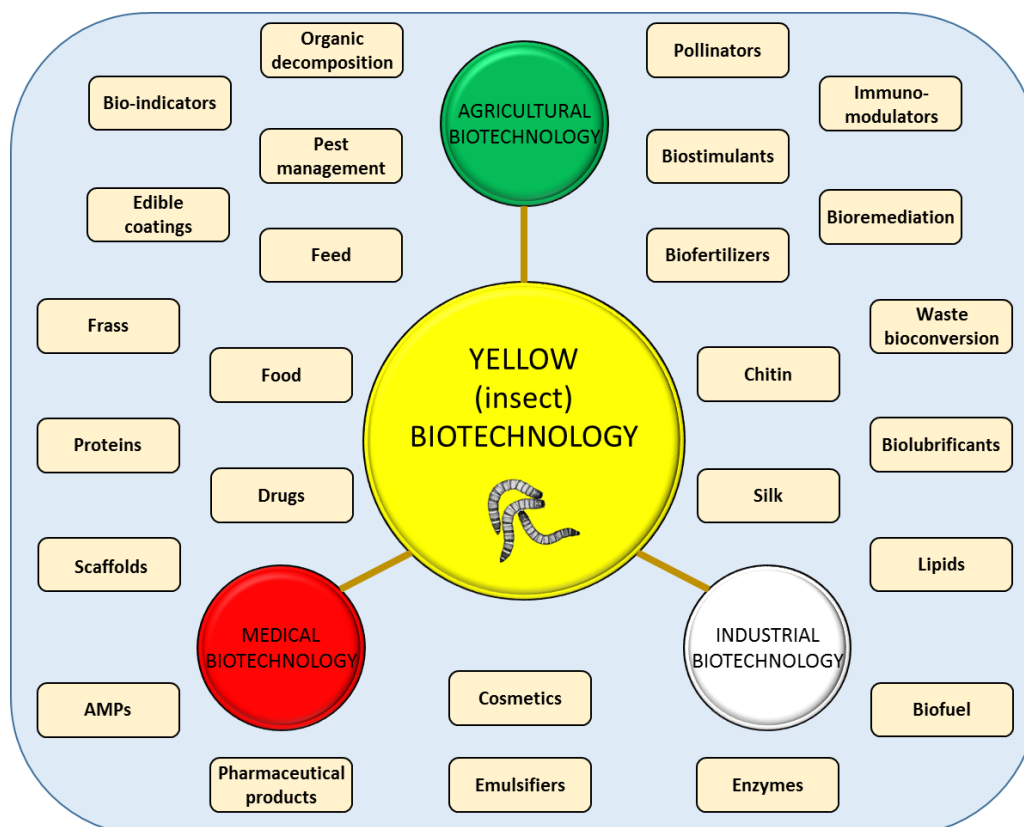


Figure 2. Overview of services and products offered by insects within the yellow biotechnology concept.

Starting from the rearing step, insects might offer a novel alternative strategy for waste management and disposal. Indeed, thanks to the complex microbiota living in their gut, several insect species have shown ability to grow on decaying materials contributing to reducing their environmental footprint and degrading possible contaminants (Mannaa et al., 2024; Gold et al., 2018). However, although

insects can easily adapt to different diets, defining a suitable feedstock appears crucial for maximising the rearing output in terms of biomass production yield, waste conversion efficiency and nutritional quality of the final product (Rossi et al., 2024). Harvested insects can then be used for producing animal feed or human food, or as initial biomass for extracting relevant biomolecules. When food and feed is the goal, several traditional (milling, drying, blanching, fermentation, marination, etc.) as well as novel processing technologies (e.g. high hydrostatic pressure, pulsed electric field, ultrasounds, cold atmospheric processing plasma) can be applied (Ojha et al., 2021). The aim of these technologies is to ensure high microbial and chemical safety, while keeping nutritional and organoleptic quality. However, although whole, processed or unprocessed, insects might potentially be consumed by humans and other animals, some limitations, mainly related to the scarce consumers' acceptance, sensory and textural problems or unbalanced/uncorrected nutritional composition, can be relevant. Fractionation technologies such as defatting, protein isolation, chitin removal, might therefore be needed in order to obtain more suitable and homogeneous ingredients (Smets et al., 2020; Psarianos et al., 2023; Rossi et al., 2023). Such ingredients can be kept within the food and feed chain or be used elsewhere. Agriculture, medicine and industry are the economic sectors that most benefit from insect-derived products.

Chitin, which in food and feed may act as prebiotic and immune stimulant, regulating the digestion and absorption of several macro and micronutrients and promoting the selection of positive gut microbiome (Kipkoech, 2023), whether extracted and converted into chitosan, could be used in agriculture, food industry, medical and cosmetic industry, or for environmental remediation (Rossi et al., 2023). Its application includes: fertilisation and plant health improver, emulsifier and edible coating production, production of scaffolds, wound dressings and drug capsules, production of hair conditioner, active face masks, sunscreens, dental fillers, make-up and toothpaste, water purification and pollutants removal (Abidil et al., 2020; Rehman et al., 2023; Santos et al., 2020; Almeida et al., 2020).

Similarly, while lipids in insects used for food and feed can represent an important energetic source and explicate health promoting activities (Goncalves and Aguilar, 2021), after extraction they might be used as food/feed additives, emulsifiers, lubricants, cosmetics and energetic components for biodiesel production (Lorrette and Sanchez, 2022; Manzano-Agugliaro et al., 2012; Zheng et al., 2013; Suryati et al., 2023). Alternative uses also include production of immune-modulators (Suryati et al., 2023), seed protection components (Marusich et al., 2020), plant growth stimulators (Ruban et al., 2021), nanocapsules for drugs and pesticides delivery (Chou et al., 2019) and stabilisers (Xu et al., 2021).

Proteins from insects are instead mainly used for food and feed purposes (Hasnan et al., 2023). However, when extracted and purified, they might be used as natural additives in traditional food products (Hasnan et al., 2023; de Castro et al., 2018; Gravel and Doyen, 2020) or as antioxidants (Cermenon et al., 2022), anticoagulants (Zhang et al., 2023), anti-inflammatory (Hall et al., 2020; Rivero-Pinto et al., 2024), and antimicrobial (Yi et al., 2014) components in medicine. Concerning the antimicrobial properties, insects are well known for being the most interesting source of antimicrobial peptides (AMPs). Such substances, which allow insects to grow on a wide range of highly contaminated substrates (Vogel et al., 2018), can be produced by insects themselves or by symbiotic microorganisms and might be integrated in food/feed formulations, acting as preservatives, or in medical and veterinarian drugs (Rai et al., 2016; Wang et al., 2016; Van Moll et al., 2022). The use of AMPs has also been proposed in agriculture as plant protectors and for disease vectors control (Yi et al., 2014). Alternative applications of insect proteins include: production of medical scaffolds (Mao et al., 2024; Sun et al., 2021; Bhattacharjee et al., 2017; Melke et al., 2016), production of biodegradable edible coatings with antimicrobial and antioxidant properties (Weng et al., 2023) and generation of cosmetic products with hydration, anti-aging and anti-inflammatory activities (Almeida et al., 2020). Insect-derived proteins have also been described as a source of nitrogen-rich fertilisers (Przemieniecki et al., 2021) and biostimulants for improving plant growth and nutrient assimilation (Szompá et al.,

2023; Baltazar et al., 2021). Finally, insect proteins might also be applied as enzymes source (Vilcinskas, 2015), alternative to the microbial enzymes, which can be used in the food industry (amylases, peptidases, laccases, pectinases and lipases are often used in food production) (Mika et al., 2013) as well as in the industrial biotechnology (protease, peroxidase, β -glucosidase, laccase, xylanase and mannanase are often used for degrading wood material, producing detergents and cleaning products, and hydrolyse polysaccharides) (Fischer et al., 2013; Kannan et al., 2019). Insect-derived enzymes can also be utilised in crop protection and organic matter degradation (Merzendorfer, 2013).

Therefore, insects might represent important organisms for ensuring a more sustainable future. However, although insects (and derived materials) represent the main product of an insect rearing facility, the yellow biotechnology is not solely limited to their application. Important products of the insect production are indeed represented by substrate residuals, insect excrements, exuviae and dead insects (altogether referred as frass). Although they represent actual waste of the insect production, they might still have interesting applications such as biofertilisers, soil amending or plant growing media (Lopes et al., 2022; Barragan-Fonseca et al., 2022). Moreover, as they contain chitin, microorganisms and bioactive compounds (AMPs, plant hormones, alkaloids, humic substances, phenols and enzymes), they have also been described as immune-stimulators for plants, acting as natural replacements of agrochemicals and chemical pesticides (Poveda, 2021; Barragan-Fonseca et al., 2022). Alternative uses of frass include chitin extraction, biochar or biomethan production, animal feed production, insect and earthworm growth or microbial fermentation for enzymes production (Purkayastha and Khanal, 2024; Yildirim-Aksoy et al., 2020; Lopes et al., 2022; Munoz-Seijas et al., 2024; Poveda, 2021).

Therefore, insects can provide a wide range of services, which go far beyond the traditional applications. A full understanding of such services is needed for pursuing the objective of promoting insects in the future economy, reducing the pressure on traditional resources and embracing a full transition toward the circular bioeconomy.

In this thesis, we will mainly focus on the more traditional (among the innovative) applications of insects, i.e. insects as bio-converter of waste and insects as novel food and feed source.

2.2 Insects as a tool for waste bioconversion

More than 2.2 billion tonnes of solid waste are produced globally every year (U.N., 2024b). Out of this, roughly 38% is managed in an environmentally unsafe manner, leading to human diseases, flooding, GHG production and environmental pollution (Abubakar *et al.*, 2022). Traditional controlled waste management practices include landfill and incineration. Although such techniques are preferred over the uncontrolled open dumping, they still present several environmental and health issues (Giusti, 2009). Greater benefits are instead associated with practices such as composting, mechanical biological treatment and anaerobic digestion. These methods enable the recovery of energy and nutrients from the organic fraction of the waste, which can then be used as new raw materials for other human activities (table 2). However, although these last technologies have allowed to make a big step toward the circular economy, they can still represent a threat to the environment when not applied correctly (Halkos and Petrou, 2016).

Table 2. Pros and cons of several waste management technologies.

Technology	PROS	CONS
Open Dumping	cost-effective	leachate; water contamination; toxic gasses release; diseases and pests spreading; wildlife and ecosystems damage; bad smell; risk of fires and explosions
Incineration	waste reduction; energy generation; space-saving; stable residual generation; pathogens and vectors destruction; no leachate and groundwater contamination	GHGs production; heavy metal and particulates release; high operation costs; recycling reduction; resource destruction; health and environmental risks

Landfilling	effective; land reclamation; cost-effective; stimulation of recycling; cleanliness	GHGs production; high land occupancy; leachate; groundwater pollutions; deforestation; unpleasant smell; pathogens and pests proliferation
Composting	GHGs minimisation; soil and groundwater pollution reduction; nutrients recovery	high costs; unpleasant smell and appearance; time-consuming; pests and diseases spreading
Anaerobic digestion	energy generation; unpleasant smell reduction; improved water quality; GHGs emission reduction; pathogens reduction; nutrients recovery	expensive; time-consuming; high land use; labour intensive; explosion risks; diseases spreading; difficulty of standardisation; limited efficiency; low product quality; generation of H ₂ S, NH ₃ , CO ₂ , SO ₂ , NO _x and VOCs; risks of toxic spills; necessity of pre-treatments
Thermochemical conversion (pyrolysis, combustion, hydrothermal liquefaction, gasification, torrefaction)	fast process; GHGs emission reduction; energy generation	high specific material; high costs; difficulty of standardisation; difficult upscaling; need for continuous control
Insect bioconversion	fast; nutrient recovery; GHGs emission reduction; pathogens and pests control; fertilisers and food/feed production	labour intensive; high costs; absence of standardisation; need for pre-treatment; need for frass post-composting; high bio-waste specificity; still limited knowledge

Insects as well as other arthropods have shown great ability to grow on decaying organic materials, indicating that they could offer a potential new strategy for waste bioconversion. The concept behind using insects for waste management originates from the idea that insects can convert waste and by-products while generating novel protein-rich biomass for food, feed or other biotechnological purposes (figure 3). Bioconversion residuals (i.e. frass) may then be used as fertilisers or for production of bio-stimulants or other active compounds acting as health-promoters in plant and allowing recovery and reintroduction of nutrients within the food chain (Mannaa *et al.*, 2024).

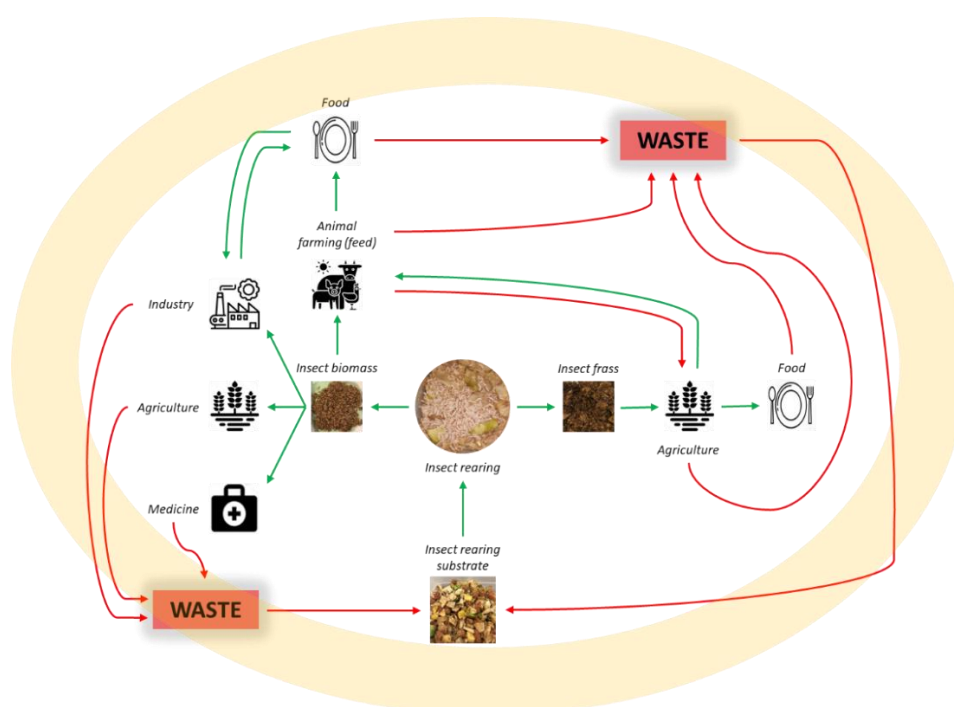


Figure 3. Example of application of insects for converting waste within the circular bioeconomy framework.

Several insect species including *Hermetia illucens*, *Musca domestica*, *Tenebrio molitor*, *Zophobas morio*, *Alphitobius diaperinus*, *Acheta domesticus*, *Gryllobes sigillatus*, *Locusta migratoria* and *Schistocerca gregaria* have been described as effective in bioconverting organic waste (Surendra *et al.*, 2020). Flesh fly (*Sarcophaga dux*) and blow fly (*Chrysoma putoria* and *Lucilla sericata*) have also been

studied; however, their economic and scientific significance is comparatively lower (Siddiqui *et al.*, 2024). Despite the long list of insect species potentially suitable for waste bioconversion, most attention is focused toward sarcophagous insects from the Diptera order, with the black soldier fly (*Hermetia illucens*) larvae (BSFL) being the most prominent. Recent studies have shown the extreme adaptability of this species to a wide range of waste, from food waste to sludge and manure (Surendra *et al.*, 2020). The dietary plasticity of this species is mainly attributed to the wide range of symbiotic microorganisms living in its gut (Gold *et al.*, 2018; Muurmann *et al.*, 2024). Insect gut microbiota composition, which is mainly dependent on the insect's diet (Bruno *et al.*, 2019), can produce several enzymes and other substances that directly or indirectly help the insects to consume and digest the waste (Mannaa *et al.*, 2024). According to this concept, Gold *et al.* (2018) defined the insect-mediated waste bioconversion process as a combination of activities occurring within two reactors: the insect's body and the waste biomass where the insects live and operate (figure 4). Therefore, optimisation of an insect-mediated waste bioconversion process should consider the interaction between these two environments. However, unlike other traditional waste management systems, this process has two main aims, i.e. waste reduction and insect biomass production.

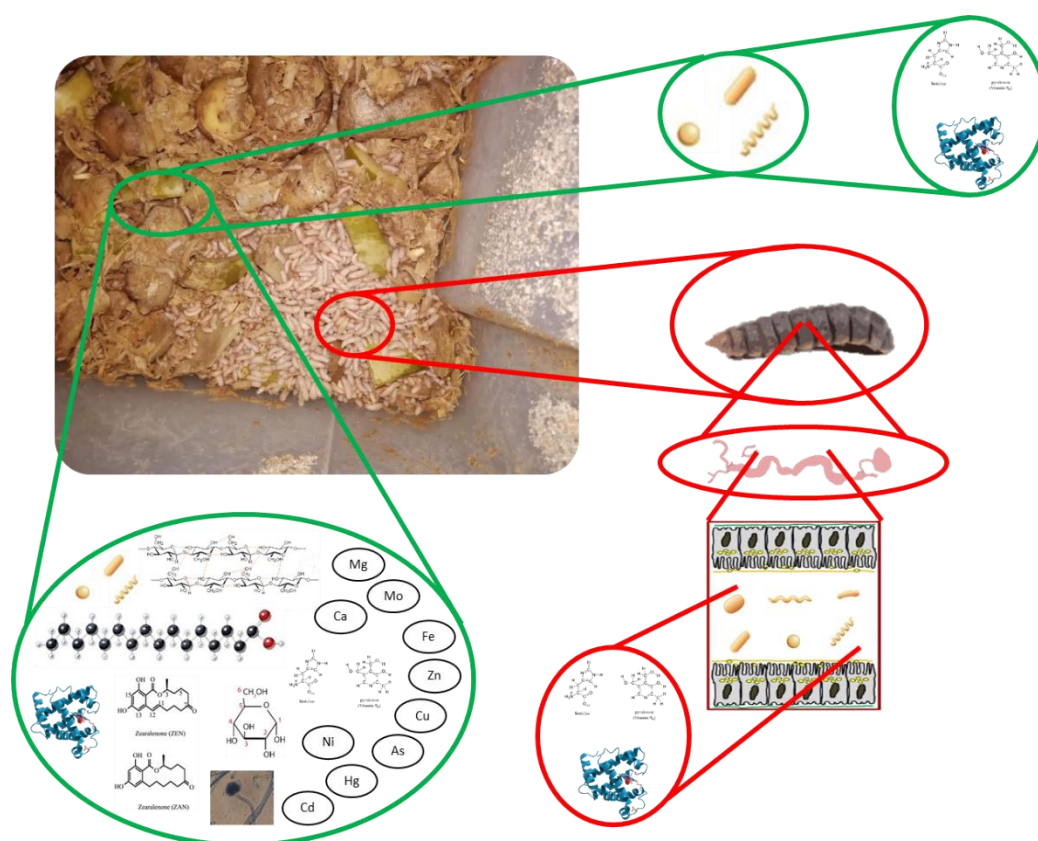


Figure 4. Interaction between the insect body bioreactor (red lines) and the waste processing bioreactor (green lines) during an insect-mediated waste bioconversion operation.

In order to optimise an insect-mediated waste bioconversion process, several aspects including temperature, relative humidity, substrate moisture, pH, texture and particle size, feed availability, feeding system, feed nutritional composition and larval density must be considered (Rossi *et al.*, 2023c; Peguero *et al.*, 2022; Surendra *et al.*, 2020; Siddiqui *et al.*, 2022; Meneguz *et al.*, 2018). All of these parameters mainly affect the larval survival and development, suggesting that they need to be addressed regardless of the waste treatment process (Barragan-Fonseca *et al.*, 2018). However, the same factors also affect feed conversion ratio, feed consumption, substrate reduction and enzymatic activity indicating that they need special attention when waste treatment is the objective. Focusing on

maximising insect production might, therefore, be the best strategy for optimising waste management. However, this cannot be generalised in all cases (Surendra *et al.*, 2020). For example, Diener *et al.* (2009) and Manurung *et al.* (2016) studied waste conversion and BSFL production under different feeding rates. They observed that, while higher feeding rate led to higher and faster insect growth, the substrate consumption was significantly lower than when low feeding rates were applied. Insects are indeed characterised by the compensatory mechanism, according to which they can prolong the development time, increase the feed ingestion rate or increase the overall amount of feed ingested in response to crowding or low dietary nutritional value (Barragan-Fonseca *et al.*, 2018). Crowding and larval aggregation have also shown to be responsible for temperature rise, which in turns affects the enzymatic activity, resulting in higher feed digestion and assimilation (Bonelli *et al.*, 2019; Paz *et al.*, 2015). Similarly, enhanced feed consumption was detected when limited feed or low quality feed was offered to the larvae (Barragan-Fonseca *et al.*, 2018), suggesting that reducing feed quantity and quality, or increasing larval density, could be interesting starting points for optimising the waste conversion performances.

However, in both cases, reduction of insect biomass production should be considered. Although it might appear secondary in a waste management process, it is actually extremely important in order to ensure high affordability and circularity of the overall system (Lalander *et al.*, 2019). Indeed, the main concept behind the use of insects as waste management tool is to convert low quality material in high quality biomass (Kee *et al.*, 2023). Additionally, as longevity and fecundity of larval adults are primarily related to larval fitness (Barragan-Fonseca *et al.*, 2018), feeding BSFL with low quality diets might result in small and low active adults. Therefore, defining the optimal parameters in terms of feed quality and quantity, larval density and feeding rate will be of primary importance in any insect rearing or insect-mediated waste bioconversion facility.

Further considerations should be given to the possibility of adapting an insect population to specific substrates and rearing conditions. Artificial selection and genetic improvement could therefore be applied on insects for developing specific rearing lineages that are better able to combine biomass production with waste bioconversion processes (Zhan *et al.*, 2020; Fowled and Nensen, 2019).

2.3 Insects as food and feed

The current food system is responsible for producing approximately 26% of the total anthropogenic GHG emissions, 32% of the global terrestrial acidification and 78% of eutrophication (Poore and Nemecek, 2018). Additionally, about 1.4 billion ha of land (~ 87% of the arable land) and 2.8 trillion m³ of freshwater (~ 70% of the total freshwater withdrawal) are currently used for food and feed production (FAO, 2022a; FAO, 2021). Recent analyses have shown that shifting from a conventional diet to an omnivore-optimised diet including limited amount of animal products may reduce the overall environmental footprint of the food system by 60-80% (Mazac *et al.*, 2022). Additionally, if an animal-free diet is adopted, it could result in 3.1 billion fewer hectares of land use, a reduction of 6.6 billion metric tons of CO₂-eq emission, 50% less acidification, 49% less eutrophication and 19% less water consumption (Poore and Nemecek, 2018). However, despite these data, an animal-free diet may not meet the nutritional requirements of people, requiring nutritional integrations, which would inevitably lead to a higher environmental impact (Parodi *et al.*, 2018).

Novel foods, including microalgae, microbial proteins, mycoproteins, cultured meat, cultured milk, mussels, sugar kelp, and edible insects, have been suggested as a strategy to meet the human nutritional requirements while ensuring a lower environmental impact of the food system (Parodi *et al.*, 2018; Mazac *et al.*, 2022). Edible insects have attracted significant interest, as they are able to produce high quality protein while keeping the environmental impact low (van Huis *et al.*, 2021). Environmental impact of edible insects strictly depends on species, rearing substrate/feed and environmental conditions. Energy needed for warming up (or cooling down) the rearing room,

ventilation and lightening represent the main environmental parameters affecting the environmental impact of insect production (Baiano, 2020). On the other hand, feeding substrate is extremely important, with decaying substances such as waste and by-product representing the most sustainable rearing materials, while commercial animal feed being environmentally unsustainable (Smetana *et al.*, 2021). However, despite these variables, insects have shown lower GHG emission, lower water and land use, higher feed conversion ratio (FCR) and higher edible percentage than traditional animals (Illa and Yuguero, 2022; de Carvalho *et al.*, 2019; Lin *et al.*, 2023). Moreover, if edible insects are promoted as animal feed, there could be indirect positive effects, such as more agricultural land available for traditional food production (Rivas-Navia *et al.*, 2023). A comparison of the environmental impact between insects and traditional meat products is presented in table 3.

Table 3. Environmental impact for producing 1 kg of edible proteins from different animals (adapted from Ros-Baro *et al.*, 2022; Oonincx *et al.*, 2010 and FAO, 2013).

Food item	Water footprint (m ³ /kg edible protein)	Land use (m ² /kg edible protein)	GWP (kg CO ₂ eq/kg edible protein)	Energy use (MJ/kg edible protein)
Pig meat	57	47-64	21-53	95-236
Chicken meat	34	42-52	18-36	80-152
Beef meat	112	144-258	75-170	177-273
Mealworms	23	18	15	170

The idea of using insects as food is not a novel concept. Ancient scriptures have suggested that insects were already eaten by humans in prehistoric times, and ancient Romans and Greeks considered them as a delicacy (Baiano, 2020). However, the custom of eating insects disappeared with time and repugnance has appeared in Western countries, where insects are no longer part of the food culture (Sogari *et al.*, 2023a). Food neophobia and disgust are indeed the main constraints limiting the exploration of insects as food on a large scale in the West (de Carvalho *et al.*, 2019). On the other hand, higher acceptability is usually observed in tropical countries (Baiano, 2020; Omuse *et al.*, 2024), although the increasing westernisation is gradually changing this trend (van Huis *et al.*, 2021).

Currently, more than 2 billion of people consume insects worldwide and more than 2200 species, belonging to Coleoptera (32%), Hymenoptera (15.5%), Lepidoptera (15.2%), Orthoptera (14.1%), Hemiptera (11.4%), Isoptera (3.4%), Odonata (2.4%), Diptera (1.8%), Dictyoptera (1.7%) orders are considered edible (Omuse *et al.*, 2024). People who traditionally consume insects have developed preparation methods that enhance their sensorial properties (van Huis *et al.*, 2021). Health promoting effects connected to their consumption have also been discovered (Nowakowski *et al.*, 2021; Rivas-Navia *et al.*, 2023; Zhou *et al.*, 2022; Aguilar-Toala *et al.*, 2022; Acosta- Estrada *et al.*, 2021). Processing of insects in unrecognisable forms such as paste and flour or extract proteins and lipids to integrate in traditional food products has been suggested as a viable method for promoting higher exploitation of edible insects in western countries (Rai *et al.*, 2023; Kozlu *et al.*, 2024). Several processing methods, including chilling, freezing, blanching, boiling, roasting, smoking, cooking, drying, marination, milling, fermentation, fractionation and extraction as well as high pressure, cold atmosphere plasma, pulsed electric field, ohmic heating and ultrasounds have therefore been investigated (Ojha *et al.*, 2021). Each operation can have both positive and negative effects on the quality and safety of the final product, whereby the choice of the processing technology and of the entire processing pathway should consider different aspects, including initial material, final product and processing parameters (Liceaga, 2021). Different processing will therefore result in different final products. Each product is characterised by specific properties in terms of chemical, physical and biological safety as well as nutritional and organoleptic quality. Packaging and storage are also crucial for preserving these properties and delivering a quality product to the final consumer or the food operator (Rossi *et al.*, 2023b). Indeed, as the current direction of the edible insect food industry is to integrate insect-based ingredients in traditional food preparation, the product placed on the market should ensure the highest quality level

in order to perfectly supplement or replace ingredients traditionally used in the food preparation (Sogari *et al.*, 2023a). Selection of the most appropriate packaging and storage conditions should therefore consider quality and safety of the material as well as food properties, applied processing, final use and desired preservation time. However, only a few studies on the packaging and storage of edible insects and their products have been published so far. An overview of these studies is provided in table 4.

Table 4. Overview of studies investigating packaging and storage of edible insects and products thereof. Adapted from Rossi *et al.* (2023b).

Species	status	packaging	storage	reference
<i>Alphitobius diaperinus</i>	Whole raw	--	-20°C	Wessels <i>et al.</i> , 2020
<i>Tenebrio molitor</i> <i>Velarifictorus asperses</i> <i>Protaetia brevitarsis</i> <i>Allomyrina dichotoma</i>	Whole blanched	--	+4°C	Son <i>et al.</i> , 2016
<i>Tenebrio molitor</i>	Whole raw Whole pan fried Whole freeze dried	--	+4°C	Kim <i>et al.</i> , 2015
<i>Tenebrio molitor</i> <i>Schistocerca gregaria</i>	Whole blanched	Plastic bags Normal air or MAP (30% CO ₂ , 70% N ₂)	+4°C	Flenka <i>et al.</i> , 2017
<i>Hermetia illucens</i>	Whole blanched Whole fermented	Vacuum packaging/ Fermentation	+25°C +15°C +4°C	Van Campenout <i>et al.</i> , 2021
<i>Termites (NS)</i>	Whole raw	Polyethylene bags	+25°C	Nightingale and Ayim, 1980
<i>Ruspolia differens</i>	Whole raw Oven roasted	--	Light/dark Vacuum/oxygen +25°C / -2°C	Ssepuuya <i>et al.</i> , 2021
<i>Gryllus bimaculatus</i>	Whole deep fried	PET/PE bags vacuum or non-vacuum	+25°C	Gan <i>et al.</i> , 2022
<i>Ruspolia nitidula</i>	Whole dried Whole roasted	Plastic (PET) Opaque/transparent Vacuum/oxygen	+25°C +4°C -20°C	Ssepuuya <i>et al.</i> , 2016
<i>Rhynchophorus phoenicis</i>	Whole boiled Whole roasted Whole frozen Whole smoked and dried	Plastic bags	+25°C +4°C -18°C	Tincheau <i>et al.</i> , 2013
<i>Ruspolia nitidula</i>	Whole boiled, roasted and dried	Vacuum opaque plastic bags	+25°C	Ssepuuya <i>et al.</i> , 2017
<i>Tenebrio molitor</i>	Whole blanched	Plastic bags	+3°C	Vandeweyer <i>et al.</i> , 2017
<i>Gynanisa maja</i> <i>Gonimbrasia zambesina</i> <i>Macrotermes falciger</i>	Whole dried	Plastic bags	+25°C	Kachapula <i>et al.</i> , 2018
<i>Imbrasia belina</i>	Whole dried	Plastic bags	+25°C	Mpuchane <i>et al.</i> , 2000
<i>Gryllodes sigillatus</i>	Whole boiled and frozen Whole boiled and dried Whole boiled and smoked	Plastic bags and glass tubes	+25°C +3°C -20°C	Vandeweyer <i>et al.</i> , 2018
<i>Encosternum delegorguei</i>	Whole fresh Whole cooked	Plastic bags/grain bags/wood baskets	+25°C	Musundire <i>et al.</i> , 2016
<i>Tenebrio molitor</i>	Whole blanched and marinated	Glass becker	+4°C	Borremans <i>et al.</i> , 2018
<i>Bombyx mori</i> <i>Omphisia fuscidentalis</i> <i>Gryllus bimaculatus</i>	Whole frozen	--	-4°C	Kurdi <i>et al.</i> , 2021
<i>Rhynchophorus phoenicis</i>	Dried and pulverised (powder)	Plastic bags	+4°C -18°C	Womeni <i>et al.</i> , 2012
<i>Gryllus bimaculatus</i>	Died and pulverised (powder)	AL/PET/LLDPE	+40°C +35°C +25°C	Kim <i>et al.</i> , 2016
<i>Tenebrio molitor</i> <i>Alphitobius diaperinus</i>	Steamed, pulverised and pan-fried (paste) Stir fried, pulverised and pan-fried (paste)	PA/EVOH/PA/PE bags Normal air or MAP (60% CO ₂ , 40% N ₂)	+4°C	Stoops <i>et al.</i> , 2017
<i>Tenebrio molitor</i>	Dried and pulverised (powder)	Cylindrical plastic vials	+26°C	Rumbos <i>et al.</i> , 2020

<i>Alphitobius diaperinus</i>	Dried and pulverised (powder)	Cylindrical plastic vials	+26°C	Rigopolou <i>et al.</i> , 2023
<i>Acheta domesticus</i>	Blanched, dried and pulverised (powder)	Polypropylene bags Polyethylene bags Plastic containers	+25°C +4°C	Kamau <i>et al.</i> , 2018a
<i>Acheta domesticus</i> <i>Hermetia illucens</i>	Blanched, dried and pulverised (powder)	Polyethylene bags	35°C	Kamau <i>et al.</i> , 2018b
<i>Hermetia illucens</i>	Blanched, boiled, dried and pulverised (powder)	Polypropylene bags Polyethylene bags Polyamide bags	+25°C +4°C	Kamau <i>et al.</i> , 2020
<i>Gryllus bimaculatus</i>	Blanched, boiled, dried and pulverised (powder)	Polyethylene bags	+40°C +25°C +4°C	Lee <i>et al.</i> , 2020
<i>Tenebrio molitor</i>	Steamed, milled and mixed with additives (paste)	Polypropylene containers	+4°C -21°C	De Smet <i>et al.</i> , 2019
<i>Tenebrio molitor</i>	Blanched, milled and fermented (paste)	Falcon tubes	+35°C	Borremans <i>et al.</i> , 2018
<i>Tenebrio molitor</i>	Blanched, milled and mixed with additives or fermented (paste)	Falcon tubes	+35°C	Borremans <i>et al.</i> , 2019
<i>Tenebrio molitor</i>	Blanched, milled and mixed with additives or fermented (paste)	Falcon tubes	+4°C	Borremans <i>et al.</i> , 2020
<i>Tenebrio molitor</i> <i>Acheta domesticus</i>	Rinsed, boiled, dried, pulverised and fermented (powder) Boiled, dried, roasted, pulverised and fermented (powder)	--	+30°C +5°C	Klunder <i>et al.</i> , 2012
<i>Tenebrio molitor</i>	Boiled, dried, milled (powder)	Paper bags Plastic containers Vacuum wrapped foils	+23°C +5°C	Kouril <i>et al.</i> , 2022
<i>Tenebrio molitor</i>	Oil extracted from dried, roaster and milled insects	--	+50°C	Jeon <i>et al.</i> , 2016

* NS, non-specified

Similar considerations apply when insects are proposed as feed, although fewer issues related to the consumer acceptance are usually encountered (Zou *et al.*, 2024). Insects are already part of the natural diet of several animals (Sogari *et al.*, 2023b) and their use in feed is considered a way to stimulate the animal's natural behaviour (Gasco *et al.*, 2019). Nevertheless, although using insects as novel feed rather than as novel food may seem technically easier, challenges linked to high price, low standardisation, low volume, low palatability, presence of potentially indigestible compounds and potential rejection by the animal need to be considered (Gasco *et al.*, 2023).

Several studies testing insects as feed for farmed animals have been carried out. These studies have suggested that partial or full replacement of traditional feed sources such as soybean meal and fishmeal is possible (Rossi *et al.*, 2024a), albeit several aspects such as insect species, development stage, nutrient composition, potential health effects, kind of meal (whole or defatted) and animal requirements, should be addressed (van Huis and Gasco, 2023; Veldkamp *et al.*, 2022; Hancz *et al.*, 2024).

2.4 Legislative landscape of edible insects in the circular bioeconomy

Although edible insects have shown great potential in promoting a transition from the traditional linear economy to a circular (bio)economy, legislations may represent a significant hurdle to the full exploitation of their potential. Legislation on edible insects in the circular bioeconomy should consider both their role in waste management and their use as food and feed.

Insects as waste management tool

Although several insect species have shown great potential for growing on biological waste (section 2.2), legislators are usually conservative and often prohibit the use of waste in insect rearing (table 5). For example, in the EU, insects are classified as farmed animals, and their production must comply

with the same restrictions that apply to other livestock (Bosch *et al.*, 2019). The feed permitted for insects is the same as that allowed for other farmed animals under EU regulations (Reg. (EC) 178/2002, Reg. (EC) 767/2009, Reg. (EC) 1069/2009, Reg. (EU) 68/2013). Therefore, insects can only be fed with material safe for human, animal and environment (Van Raamsdonk *et al.*, 2023). Manure, food waste generated from restaurant, catering services and household, animal carcasses and meat/fish unfit for human consumption, are therefore not allowed. On the other hand, vegetable and non-contaminated agriculture waste can be used (Rossi *et al.*, 2024a).

Less strict legislation is applied in Asian and African countries, where governments tend to incentivise farmers to use manure and food waste for rearing insects, as long as the obtained insects are safe for being used as animal feed (Bai *et al.*, 2021; Niassy *et al.*, 2022). Similar considerations are also valid in countries from Centre and South America, where no clear feed ban is present (Allegretti *et al.*, 2017). Canadian government is relatively open to the idea of using insects for recycling food waste (Ominski *et al.*, 2021), while the US government appears to be more conservative (Leib *et al.*, 2016) on this matter. Specifically, according to the US legislation, every animal species should be considered separately and a list of feed allowed for each species is generally available. However, insects are not mentioned as farmed animals in the US legislation, suggesting that food and agriculture waste (but not manure) may be used, provided they do not pose a risk to animal health (Lahteenmaki-Uutela *et al.*, 2021).

Table 5. List of waste and by-products allowed/not-allowed for rearing insects in different countries. Adapted from Rossi *et al.* (2024a).

Country	Manure	Vegetable food waste	Animal food waste	Reference
Europe	X	✓	✓/X	Van Raamsdonk <i>et al.</i> , 2023
Africa	✓	✓	✓	Niassy <i>et al.</i> , 2022
Asia	✓	✓	✓	Bai <i>et al.</i> , 2021 Mukherjee <i>et al.</i> , 2023
Australia	X	✓	X	https://www.sfmca.com.au/integrity-systems
North America	X	✓	X	Ominski <i>et al.</i> , 2021 Lahteenmaki-Uutela <i>et al.</i> , 2021
South America	✓	✓	✓	Allegretti <i>et al.</i> , 2017

Insects as food and feed

Generally, legislators are open toward the novelty of insects as food and feed, as long as the insects are safe and do not represent threats for human and animal health.

In the EU, insects are considered novel food and therefore need to go through a safety evaluation before entering the market (Reg. (EU) 2283/2015). To date (August 2024), four edible insects have been allowed in the European market, which include:

- *Tenebrio molitor* larvae (whole dried; whole frozen)
- *Locusta migratoria* (whole frozen; whole dried; powder)
- *Acheta domesticus* (whole frozen; whole dried; partially defatted powder)
- *Alphitobius diaperinus* (whole frozen; whole dried; paste; powder)

According to the current European legislation, when one novel food is authorised, parties other than the applicant are also allowed to commercialise the same product (in the same status) without presenting a new application (Sogari *et al.*, 2023a). It may favour a better promotion of edible insects in the European market. On the other hand, the novel food regulation also includes a data protection rule, according to which the scientific data presented by the applicant are kept secret for a five years period. Such condition, which protect the interest of the applicant, could slow down the affirmation of the new product in the market (Traynor *et al.*, 2024). It is important to underline that when a novel food is authorised, it must comply with the same legislation as any other food.

Regarding other parts of the world, the legislation is quite varied. While insects are traditionally recognised as food and not subject to specific legislation (significantly different from other food) in most of the Asian, African and Latin American countries (Abril *et al.*, 2022; Grabowski *et al.*, 2020; Lin *et al.*, 2023; Devi *et al.*, 2024), specific legislation is present in North America. According to the Canadian legislation, insects, being traditional food in third countries, can enter the market without restriction, as long as they are included in the “List of non-novel determinations for food and food ingredients” (to the date, it includes three species: *Tenebrio molitor*, *Alphitobius diaperinus* and *Gryllobates sigillatus*) (Larouche *et al.*, 2023). On the other hand, no specific restriction is present in the US, where insects as food are regulated through the Federal Food, Drug, and Cosmetic Act. According to this, edible insects should be Generally Recognised as Safe (GRAS), and should be considered as food if that is their intended use. However, to be suitable for consumption, they must be produced in compliance with all regulatory requirements of other foods. This means they should be clean and wholesome, produced and processed under sanitary conditions following the good manufacturing practices (GMP) and properly labelled. Specific authorisation is instead required when insects or insect components are used as food additive (Larouche *et al.*, 2023). Furthermore, in Australia, although insects have traditionally been consumed by Aboriginals, their legal production has never been regulated. However, following the examples of Europe, the Australian governments have recently approved three insect species (*Zophobas morio*, *Acheta domesticus*, *Tenebrio molitor*) in whole, ground or paste form. Insect production and consumption has only recently been accepted in Singapore. Sixteen insect species have been approved (*Acheta domesticus*, *Gryllobates sigillatus*, *Teleogryllus testaceus*, *Gryllus bimaculatus*, *Locusta migratoria*, *Schistocerca gregaria*, *Oxya japonica*, *Zophobas atratus*, *Tenebrio molitor*, *Alphitobius diaperinus*, *Galleria mellonella*, *Achoria grisella*, *Bombix mori*, *Protaetia brevitarsis*, *Alomyrina dichotoma*, *Apis mellifera*), although strict limitations related to life stage and rearing procedures are applied.

In most countries, use of insects as feed is instead allowed in aquaculture and pet-feed, although some limitations mainly concerning insect species, product form and insect rearing substrate, are always present (Rossi *et al.*, 2024a). Tighter restrictions are present for other animals, including pig and poultry. Feeding insects to ruminants is usually not allowed.

In the EU, live insects, insect fat and hydrolysed insect protein are allowed to be used for animal feed without any specific limitation (only hydrolysed insect protein and insect fat, but not live insects, are allowed for ruminants) (Commission Directive 82/475/EEC). However, in case of non-hydrolysed insect proteins, only 8 species (*Musca domestica*, *Hermetia illucens*, *Tenebrio molitor*, *Alphitobius diaperinus*, *Acheta domesticus*, *Gryllobates sigillatus*, *Gryllus assimilis*, *Bombix mori*) have been considered as feed so far. Their use is allowed for poultry, pig and fish (Reg. (EU) 893/2017, Reg. (EU) 1372/2021, Reg. (EU) 1925/2021), but not for ruminants (Reg. (EU) 999/2001). Dead, treated or untreated whole insects are not allowed for any animal species (IPIFF, 2024). When insects are produced for feed, the general feed legislation applies (Reg. (EC) 183/2005, Reg. (EC) 767/2009; Commission Directive 2002/32).

In the US, insects are allowed as feed after being approved by the Association of American Feed Control (AAFCo) and being generally recognised as safe (GRAS). To the date, only *Hermetia illucens* larvae have been approved as feed in fish, poultry and swine farming (Lahteenmaki-Uutela *et al.*, 2021), while dried black soldier fly can be used in dog feed, although it is not permitted for cats, lactating female dogs or puppies (Larouche *et al.*, 2023). In Canada, there are no restrictions on insect-based pet feed (Valdes *et al.*, 2022), although compliance with the Good Manufacturing Practices (GMP) is required (Larouche *et al.*, 2023). On the other hand, specific authorisation granted by the Canadian Food Inspection Agency (CFIA) is required when insects are used for feeding food-producing animals (Alagappan *et al.*, 2022). To the date, *Hermetia illucens* larvae are the only authorised insects for use as feed in Canada; however, they can only be used for feeding fish and poultry (Lahteenmaki-Uutela *et al.*, 2021).

No specific limitations are present in Latin America, Asian and African countries (Valdes *et al.*, 2022; Lee *et al.*, 2022). However, in all cases, the safety of the fed animals must always be ensured and no

materials posing a risk for farmed animals or humans should be used (Sogari *et al.*, 2019; Niassy *et al.*, 2018; Niassy *et al.*, 2022). Specific legislation is in place in Japan (where every new feed should be authorised by the Ministry of Agriculture, Forestry and Fisheries), in North Korea and Malaysia (where insects and all animal proteins are prohibited as feed).

In Australia, insects as well as any other material free of veterinary drugs and not dangerous can be used for feeding animals (Lee *et al.*, 2022; Valdes *et al.*, 2022). However, raw (live or untreated) insects cannot be used for any animal, while processed insects are only forbidden for ruminants (Lahteenmaki-Uutela *et al.*, 2021).

3. Discussion

3.1 Non-destructive methods for in-line monitoring of edible insect products quality

Traditional chemical methods for analysing food and feed are destructive, time-consuming and require huge amount of chemicals (Kharbac *et al.*, 2023). Despite they are highly precise and accurate and therefore preferred over novel analytical tools, innovative spectroscopic methods are gaining interest (Hassoun *et al.*, 2020). These technologies are based on the analysis of spectra generated from the interaction of electromagnetic radiation with matter (Penner, 2017). Following the exposition of a certain material to a specific electromagnetic radiation, the chemical species contained within the material are indeed able to absorb a certain amount of energy, resulting in an electronic transition (excited status). As the new excited status is extremely instable, the absorbed energy will be re-emit, generating a new electromagnetic radiation (figure 5). According with the first law of thermodynamics (energy cannot be create or destroyed but it can only be transformed), the amount of energy emitted will always be lower than the amount of energy absorbed. The difference between these two energies is known as Stoke shift. It happens because, when a material is exposed to an electromagnetic radiation, only one small amount of the total energy transported by the radiation is absorbed, while most of it is reflected and lost (scattering). Analyse the electromagnetic spectra generated from the sample-radiation interaction as well as the amount of energy absorbed, emitted, reflected or lost, will allow to qualitatively identify and quantitatively determinate the chemical species responsible for the event.

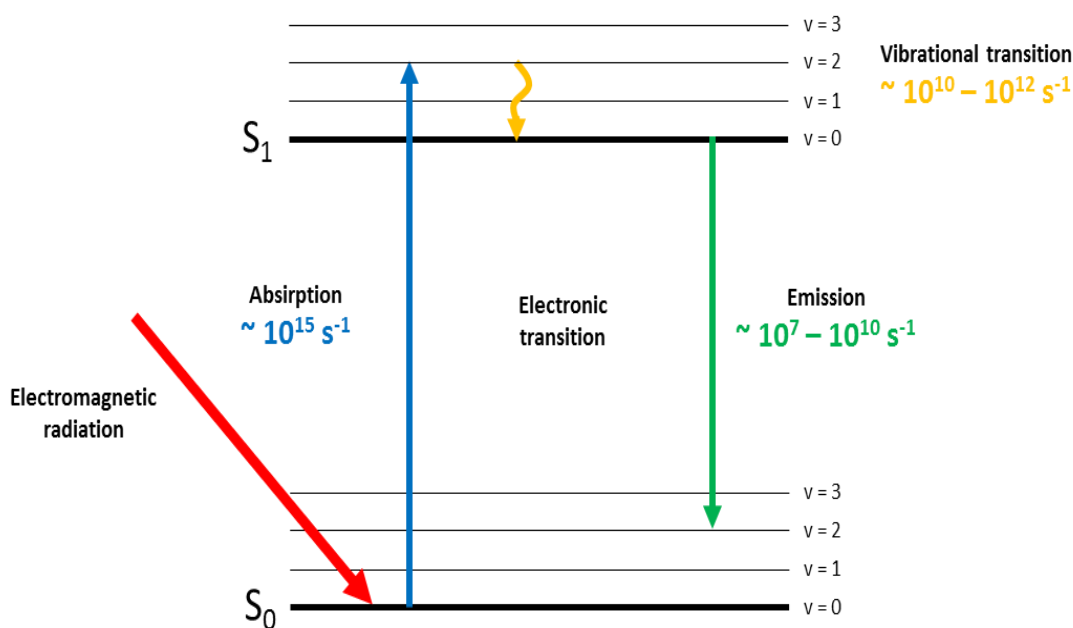


Figure 5. Example of electronic transition resulting from a chemical species exposed to an electromagnetic radiation.

Depending on the type of energy and electromagnetic radiation studied as well as on the phenomena target of the investigation, several spectroscopic techniques have been developed. An overview of these techniques is offered in table 6. Among them, fluorescence represent an attractive technique as it appears more selective and sensitive than others methods (Nelson, 2017). Fluorescence is indeed the only spectroscopic technique characterised by a double identification wavelength (excitation and emission), while the high intensity of emission allows to clearly detect highly diluted molecules.

Table 6. Overview of spectroscopic techniques used in food analyses (adapted from Zhong and Wang, 2019; Barba *et al.*, 2020; Mendes and Duarte, 2021).

Spectroscopic technique	Principle	Application in food science
UV-Vis	Electronic transition	Quality assessment (oxidation, phenolic content, antioxidant activity, colour); Detection of adulteration; Classification; Chemical characterisation; Authentication
Fluorescence	Electronic transition	Quality assessment (oxidation, phenolic content, freshness, texture, detection of mycotoxins and other contaminants, antioxidant activity); Detection of adulteration; Classification; Chemical characterisation (polyphenols, vitamins, chlorophylls); Authentication
Mid-infrared (MIR)	Electronic transition	Quality assessment (oxidation); Detection of adulteration; Classification; Authentication; Chemical characterisation;
Near-infrared (NIR)	Electronic transition	Quality assessment (phenolic content; fish freshness); Safety evaluation; Detection of adulteration; Classification; Chemical characterisation; Authentication
Dynamic light scattering (DLS)	Scattering	Quality assessment (structure, intra- and inter-molecular interactions; protein conformation)
X-ray diffraction (XRD)	Scattering	Quality assessment (crystallinity of fat and sugar, structure, molecular arrangement, gelatinisation); Chemical characterisation (fatty acids); Investigation of functional properties (oil binding capacity, solvation)
Raman	Vibrational transition	Quality assessment (pH, colour; structure); Safety evaluation; Detection of adulteration; Classification; Chemical characterisation (proximate, fatty acids); Authentication
Nuclear Magnetic Resonance (NMR)	Nuclei orientation within a magnetic field	Quality assessment (fish freshness, phenolic content, texture); Detection of adulteration; Classification; Chemical characterisation (proximate, fatty acids, organic acids, carbohydrates, phenolic); Authentication
Electron Magnetic Resonance (EMR) or Electron Spin Resonance (ESR)	Electron orientation within a magnetic field	Quality assessment (oxidation, detection of free radicals, antioxidant activity)
Atomic Absorption (AAS)	Electronic transition	Chemical characterisation (minerals); Detection of heavy metals
Atomic Emission (AES)	Electronic transition	Chemical characterisation (minerals); Detection of heavy metal; Classification; Authentication

While these methods can be satisfactory used in traditional analytical chemistry, which includes an extraction and purification of the target compound that will then be quantified, the same methodologies can also be applied on intact food (Mishra, 2022). Such operation, which significantly reduces the analysis time as well as the use of chemical reagents and sample preparation steps, allows in-line quality monitoring and simultaneous investigation of several properties of the studied sample (Kharbach *et al.*, 2023). However, due to the complexity of the food matrixes, quenching, peaks overlapping and molecular interactions might be experienced, making the results interpretation challenging (Hassoun *et al.*, 2020). In order to overcome these issues, chemometric techniques have been developed.

Spectroscopic methods already applied on edible insects are limited to NIR, which has been mainly used for chemical characterisation (Riu *et al.*, 2022; Kröncke *et al.*, 2023; Kröncke and Benning, 2023), authentication (Mellado-Carretero *et al.*, 2020), detection of adulteration (Ni *et al.*, 2024) and classification (Garcia-Gutierrez *et al.*, 2021). Recently, MIR has also be applied with the main aim of detecting adulteration of cricket powder in traditional meal flours (Alagappan *et al.*, 2024).

The research paper discussed in this section (annex 5; Rossi *et al.*, 2021) describes a first application of the fluorescence technique in the analysis of edible insect flours. The main aim of the study was to understand which chemical compounds in edible insects are responsible for raising the fluorescence signal. Five insect species belonging to the Orthoptera order and named *Acheta domesticus*, *Gryllus assimilis*, *Gryllus bimaculatus* (suborder Ensifera), *Locusta migratoria*, *Schistocerca gregaria* (suborder Caelifera) were provided by three different producers. Upon arrival, the insects were starved for 24 hours and killed by freezing at -30°C. Insects were therefore freeze-dried (3 days, -58°C, 600 mbar) and milled (knife mill, 10 rpm, 30s x2). Fluorescence spectra were recorded in triplicate by scanning the emission spectra from 260 to 700 nm (0.5 nm steps) over an excitation range between 240 and 600 nm. Front-face fluorescence, a technique that allows direct analysis of intact solid samples, minimising the inner filter effect, was applied. A three-dimensional Excitation Emission Matrix (EEM) was therefore obtained (figure 6). EEM consists of a three-dimensional representation of the fluorescence landscape (a.k.a. fluorescence fingerprint) of the studied sample. It can be mathematically described as a function of the concentration α of a fluorophore i , identified by a specific couple of excitation b and emission c wavelengths (Christiansen *et al.*, 2006):

$$EEM = \sum \alpha_i * b_i(\lambda_{ex}) * c_i(\lambda_{em})$$

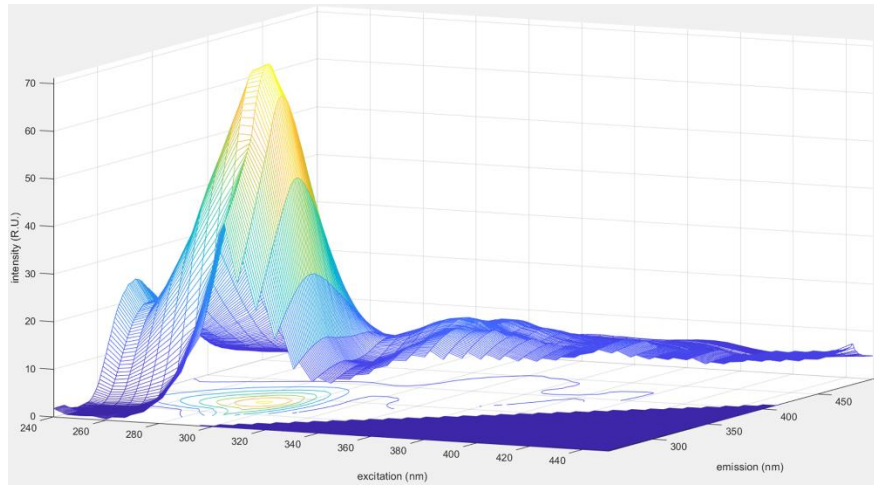


Figure 6. Excitation Emission Matrix recorded from *Acheta domesticus* flour. Adapted from Rossi *et al.* (2021).

Chemometric analysis carried out on these EEM were performed by applying the PARAllel FACtorial (PARAFAC) methodology. PARAFAC is a deconvolution method suitable for multi-dimensional data (Bro, 1997). It can be interpreted as an extension of the Principal Component Analyses (PCA), the main purpose of which is to resolve complex datasets by minimising the sum of squares of the residuals (Murphy *et al.*, 2013):

$$x_{ijk} = \sum \alpha_{if} * b_{jf} * c_{kf} + e_{ijk}$$

In the specific case of EEM, PARAFAC analysis is able to decompose the three-dimensional array describing the fluorescence landscape of the analysed samples containing the fluorophore x , in three different loadings, representing the samples i (intensity of the fluorophore within each sample), the excitation wavelengths j (excitation wavelengths where the fluorophores are located) and the emission wavelengths k (emission wavelengths where the fluorophores are located). It follows that each sample within the analysed EEM should contain the same fluorophores and all the fluorophores should be present within each sample. Additionally, as PARAFAC is characterised by unicity of the solution (differently by the traditional PCA, PARAFAC is not affected by the rotational problem), each excitation j and emission k loadings couple (and therefore each PARAFAC component) will be associated to a specific fluorophore present within the analysed EEM (Christensen *et al.*, 2006). In such a case,

whether data-linearity is also respected, the intensity of each fluorophore identified by the PARAFAC analysis can be considered as proportional to the concentration of the fluorophore within the analysed sample (Anderson and Bro, 2003).

Following the PARAFAC method, several models describing the investigated EEM were built. Model validation was carried out according to split-half analysis, core consistency, percentage of explained variance and visual observation (Anderson and Bro, 2003). An evolution of the jack-knife (leave-one-out) technique, consisting in comparing PARAFAC models built on species-specific datasets, was further applied. Based on such techniques, PARAFAC model-5 was extrapolated and further investigated. Such model described five different peaks centred at excitation/emission couples 285/333, 295/300-330, 340/390-430, 390/450, 465-470/500-550 nm. According to the theory behind the PARAFAC (i.e. each identified component is associated with one specific chemical species (Anderson and Bro, 2003)), peak identification was carried out.

Based on a literature research, the main fluorophores present in food as well as in insects were therefore identified. Fluorophores typically present in food include amino-acids, proteins, enzymes, nucleic acids, vitamins, lipids, phenolic compounds, chlorophylls, Maillard reaction products, oxidation products and contaminants (antibiotics, pesticides, mycotoxins, faecal contaminants) (Christensen *et al.*, 2006; Radotic *et al.*, 2023). Further fluorophores typically recorded in insects include pterins (Abels and Ludescher, 2003) and the protein resilin (Mouchet *et al.*, 2023). As each fluorophore is characterised by a specific excitation/emission wavelength couple, the optical properties of each fluorophore were compared with the excitation and emission spectra of the peaks detected in the obtained EEM and extrapolated through PARAFAC analysis. Several solutions containing pure fluorophores were prepared and the fluorescence spectra of each solution was recorded and alternatively added to the insect-derived EEMs. PARAFAC methodology was therefore applied and new PARAFAC models were computed. Identification of compounds responsible for each peak in the insect EEM was therefore carried out (table 7) based on the main assumption and principle of the PARAFAC methodology.

Table 7. Fluorophores identified in edible insects. Adapted from Rossi *et al.* (2021).

Fluorophores in insects ($\lambda_{ex}/\lambda_{em}$)	Verified chemical compounds	Fluorescence spectra of the chemical species ($\lambda_{ex}/\lambda_{em}$) as recorded
285/333	Tryptophan + Tyrosine	Tryptophan: 285/360 Tyrosine: 280/300
295/300-333	Tryptophan + Tyrosine + Tocopherol	Tocopherol: 295/320-330
340/390-430	Collagen + Pyridoxine + Pterins + NADH + (Schiff bases – hypothesised)	Collagen: 340/380-390 Pyridoxine: 340/390 Pterins: 340/430 NADH: 340/430-440
390/450 465-470/500-530	Hypothesised: lipid oxidation products, vitamins (vit. B2), polyphenols (phenolic acid)	--

This study represents the first example where a fully chemometric approach was applied for fluorophores identification. Although due to the high noise detected in the far-right EEM region and the low S/N ratio observed at excitation wavelengths higher than 450 nm and emission higher than 500 nm, a full characterisation of the fluorescence landscape of edible insects was not achieved, the high consistency of the obtained data suggested reliability of the applied methodology. Additionally, this study showed that the main fluorophores responsible for raising fluorescence peaks in edible insects were highly conserved among different insect species. Further analysis conducted on other edible insects (e.g. *Hermetia illucens* larvae and *Tenebrio molitor* larvae) and not included in the present study (figure 7) indeed confirmed that fluorescence in edible insects is highly conserved and could originate from a mixture of food-derived and insect-derived fluorophores. Such considerations therefore suggest that intrinsic fluorescence spectroscopy could satisfactory be applied as a method for fast detection of food adulteration.

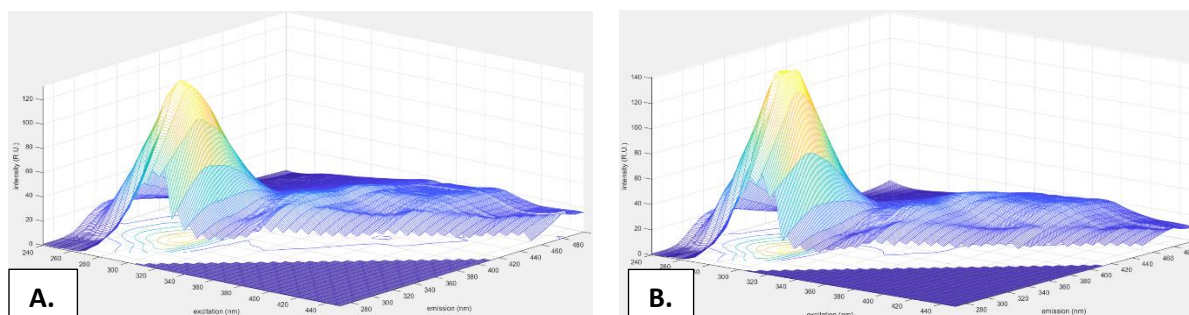


Figure 7. EEM recorded from *Tenebrio molitor* larvae flour (A) and *Hermetia illucens* larvae flour (B).

On the other hand, focusing on food-derived fluorophores detected in edible insects, a similar behaviour to traditional food was observed, suggesting that fluorescence applications in traditional food science might theoretically also be suitable for edible insects. For example, fluorescence peak typically associated with tryptophan ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 280\text{-}285/330\text{-}350\text{ nm}$) might be applied for detecting changes in the protein structure, protein oxidation, and/or modification of the external environment where the protein are located (Bußler *et al.*, 2016; Boukil *et al.*, 2022; Estevez *et al.*, 2008). On the other hand, peaks located at higher excitation/emission regions ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 330\text{-}350/400\text{-}500\text{ nm}$) might be suitable for detecting oxidation and/or browning processes, i.e. modifications occurring in food during processing and storage (Estevez *et al.*, 2008; Crobotova *et al.*, 2020; Christensen *et al.*, 2003; Dominguez *et al.*, 2022; Karoui *et al.*, 2007; Shaikh and ODonnell, 2017; Ghnimi *et al.*, 2022).

3.2 Processing and storage of insects for food applications

Food processing can be defined as “any controlled change that is made to a food to alter its eating quality or shelf life” (www.fao.org).

Food storage is the post-harvest phase of the food production chain that aims to guarantee safety and quality of the food during a certain period of time (www.fao.org).

From these definitions, we can understand that processing and storage are two critical phases of the food production chain, which are strictly connected and cannot be addressed separately. Indeed, while processing aims to improve the properties of a certain food product, storage aims to preserve such properties, ensuring that the final consumer can benefit from them.

In this section, two research papers are discussed (annex 6, Ojha *et al.*, 2024; annex 7, Rossi *et al.*, submitted-A). In these papers, application of high hydrostatic pressure (HHP) on paste originated by milling *Tenebrio molitor* larvae is investigated. Effects of such processing technology are therefore studied along a storage period of 15 (Rossi *et al.*, submitted-A) or 28 days (Ojha *et al.*, 2024), during which the pastes were kept under different conditions of modified atmosphere.

HHP can be considered as a novel non-thermal processing technology involving a fluid that, submitted to pressures in the range of 100-1000 MPa, is able to exercise a direct and uniform effect on a solid material, which is in direct contact with the fluid (Aganovic *et al.*, 2021). A schematic representation of HHP applied to a food product is offered in figure 8. HHP is governed by three main principles, e.g. the isostatic principle, the Le Chatelier’s principle and the microscopic ordering principle (Wang *et al.*, 2024). The isostatic principle (Pascal’s principle) affirms that during a HHP treatment, pressure is applied uniformly, homogeneously and instantaneously from all directions, regardless of the shape and the size of the food. The Le Chatelier’s principle instead states that when a dynamic equilibrium is disturbed, this equilibrium changes in a way that counteract the change. Therefore, in the specific case of HHP, the increase in pressure will result in a volume reduction. Finally, the microscopic ordering principle affirms that if the temperature is constant, application of pressure will result in an increasing of the molecules ordering. This principle suggests that pressure and temperatures act with opposing

forces, and high pressures are able to counterbalance the molecular disorder caused by an increase in temperature.

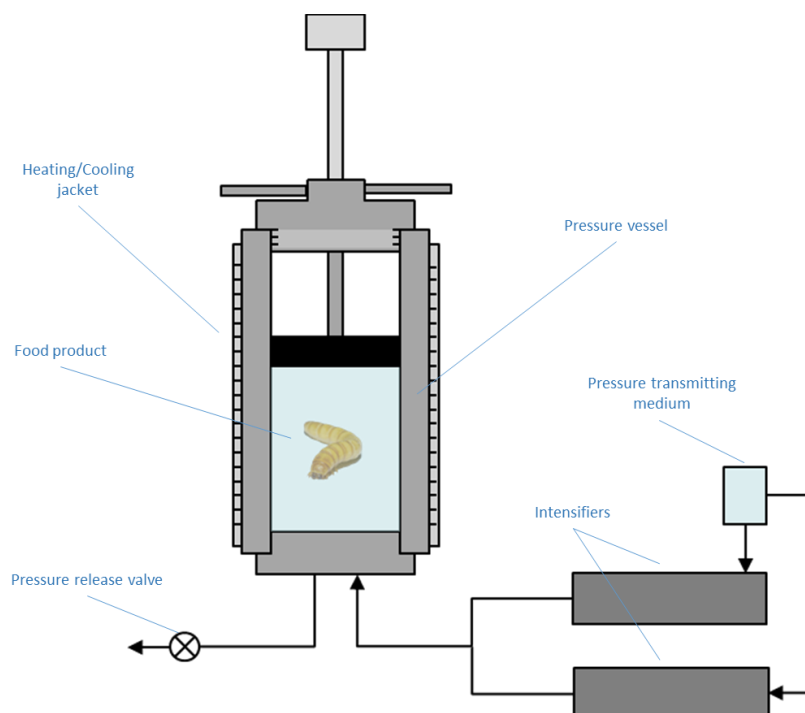


Figure 8. Schematic representation of a HHP system applied on food products.

The main applications of HHP in food technology include microbial reduction, enzymes (in)activation, shelf-life extension, protein denaturation and allergens reduction, nutrient extraction, colour stabilisation, texture modification and shift-freezing (Bermudez-Aguirre and Barbosa-Canovas, 2011; Wang *et al.*, 2016b). Due to its particular activities, it is often referred as cold-pasteurisation (Melton *et al.*, 2019). Indeed, it allows to reach similar effects than traditional pasteurisation, but without applying heat, therefore preventing the degradation of compounds responsible for colour, flavour and aroma, (Yamamoto, 2017; Mahanta *et al.*, 2021). Production of off-flavours and other substances that may negatively impact the organoleptic quality of the food will also be avoided (Wang *et al.*, 2016b). So far, only few studies investigating the application of HHP on edible insects have been published (table 8). Most of these studies investigated HHP as a method for increasing lipids and protein extraction, while few investigations have focused on HHP as a strategy for inactivate microorganisms.

Table 8. Application of HHP on edible insects. Adapted from Ojha *et al.* (2021).

Species	Product	HHP conditions	Main findings	References
<i>Tenebrio molitor</i>	whole larvae	HHP-assisted microbial inactivation (400,500, 600 MPa, 1 to 15 min)	HHP at 600 MPa were effective on reducing total microbial load after 1 min of treatment. Similar reduction was attributed at HHP carried out at 400 MPa for 15 min, while HHP at 200 MPa did not allow any significant microbial reduction	Rumpold <i>et al.</i> , 2014
<i>Hermetia illucens</i>	whole larvae	HHP-assisted microbial inactivation (250, 350, 400 MPa, 1 to 15 min)	HHP at 400 MPa allowed up to 5-log reduction of yeasts and moulds, while low reduction was detected for total viable count. HHP at 250 MPa only allowed yeasts and moulds reduction if at least 10 min treatment were applied.	Kashiri <i>et al.</i> , 2018
<i>Tenebrio molitor</i>	whole larvae	250, 300, 400, 500 MPa, 3 min, 20.5°C	HHP at 400 and 500 MPa allowed inactivation of browning enzymes but weakened the texture of the product.	Tonneijck-Srpova <i>et al.</i> , 2019

<i>Hermetia illucens</i>	live larvae	HHP-mediated killing (600 MPa, 3 min, 21°C)	HHP significantly reduced the lipid content and the microbial load, but promoted lipid oxidation	Larouche <i>et al.</i> , 2019
<i>Hermetia illucens</i>	whole larvae	400 or 600 MPa, 1.5 or 10 min, 18 °C	HHP at 600 MPa (1.5 and 10 min) and at 400 MPa (10 min) was able to significantly reduce microbial load. HHP was able to increase in vitro ruminal digestibility, but decreased the monogastric digestibility	Campbell <i>et al.</i> , 2020
<i>Acheta domesticus</i>	protein gel	HHP-assisted microbial inactivation: (a) 200, 250, 350 MPa, 1.5 to 7 min (b) 300, 325, 350 MPa, 1.5 to 7 min	HHP was able to reduce microbial load, however the reductions were lower than 5-log	Marin <i>et al.</i> , 2020
<i>Tenebrio molitor</i>	protein powder	HHP-assisted protein hydrolysis applied prior or during enzymatic hydrolysis (380 MPa, 1 min)	HHP performed during Alcalase® hydrolysis improved protein hydrolysis and in-vitro digestion of allergenic proteins.	Boukil <i>et al.</i> , 2020
<i>Tenebrio molitor</i> <i>Gryllosid sigillatus</i>	protein powder	HHP-assisted protein hydrolysis (200, 380 MPa, 1 min)	HHP induced protein denaturation and aggregation, reducing the hydrolysis degree. In mealworm, HHP improved solubility and oil binding capacity, while solubility was reduced after HHP for cricket protein.	Dion-Poulin <i>et al.</i> , 2020
<i>Protaetia brevitarsis</i> <i>seulensis</i>	defatted powder	HHP-assisted protein extraction (100, 200, 300, 400, 500 MPa, 35°C)	HHP promoted higher protein solubility with the maximum extraction yield reached at 200 MPa. Foaming capacity and stability increased with the pressure, while emulsion capacity decreased. Emulsion stability was the higher with 400 MPa treatment.	Kim <i>et al.</i> , 2021
<i>Tenebrio molitor</i> <i>Acheta domesticus</i>	whole powder	HHP-assisted defatting (500 MPa, 15 min, 30°C and 40°C)	HHP promoted higher phenolic extraction in insect powder, but the antioxidant activity did not change.	Bolat <i>et al.</i> , 2021
<i>Tenebrio molitor</i> <i>Acheta domesticus</i>	whole powder	HHP-assisted oil extraction (500°C, 15 min, 30-40°C)	HHP reduced the oil extraction yield and increased peroxide value and antioxidant activity. Phenolic content increased after HHP at 30°C, but decreased after HHP at 40°C. Monounsaturated and polyunsaturated fatty acids increased in oil after HHP, while saturated fatty acids decreased.	Ugur <i>et al.</i> , 2021
<i>Acheta domesticus</i>	protein powder treated with transglutaminase or glucose oxidase enzymes	HHP-assisted gelatinisation (500 MPa, 15 min, 25°C)	HHP did not allow protein gelatinisation after enzymatic treatment.	Urbina <i>et al.</i> , 2021
<i>Tenebrio molitor</i>	protein powder	70, 140, 210, 275, 345, 600 MPa, 5 min, 20°C	HHP promoted protein unfolding and aggregation. HHP at 600 MPa showed the strongest effects.	Boukil <i>et al.</i> , 2022
<i>Alphitobius diaperinus</i> <i>Acheta domesticus</i>	protein powder	200, 400, 600 MPa, 10 min, 20 °C	HHP reduced the microbial load and preserved the protein structure. HHP was not able to inactivate protease but increased the protein solubility.	Sweers <i>et al.</i> , 2024

The two research papers described in this section aim to: (i) investigate the ability of HHP on reducing the microbial load of fresh *T. molitor* paste stored under refrigeration by applied several MAP conditions, (ii) investigate if the use of commercial antioxidant mixtures are able to reduce the lipid oxidation trigger by HHP.

In both studies, HHP at 600 MPa for 5 minutes was applied on fresh *T. molitor* pastes packed under vacuum.

In the first study (experiment 2.1, annex 6), vacuum packages were open immediately after the treatment and the paste were further distributed within polypropylene trays sealed with

polypropylene films and stored at 4°C for 28 days. Such operations, although uncommon at industrial level (one of the main advantages of HHP is the possibility to treat already packed food), were needed because of the particular set-up of the HHP chamber available in the laboratory. Three different MAP conditions and a standard air packaging were therefore applied on treated mealworm paste (table 9) and the evolution of microbial load (total aerobic mesophilic bacterial, AM; total anaerobic mesophilic bacteria, ANM; aerobic mesophilic bacteria spores, MS; yeasts and moulds, Y&M; prevalence of *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Clostridium perfringens*), pH, colour, lipid oxidation (TBARs) and protein oxidation (fluorescence spectroscopy, λ_{ex} = 250-295 nm; λ_{em} = 270-500 nm) was carried out on 7-days intervals.

Table 9. MAP conditions applied on HHP-treated *Tenebrio molitor* pastes in experiment 2.1. Adapted from Ojha *et al.* (2023).

Sample	N ₂ (%)	O ₂ (%)	CO ₂ (%)
Air (control)	78	21	1
MAP-2	30	0	70
MAP-3	60	20	20
MAP-4	70	0	30

In the second experiment (experiment 2.2, annex 7), two commercial antioxidant mixtures were alternatively supplemented to *T. molitor* paste prior to applying HHP. Permanent vacuum packaging was ensured along the entire experiment and microbial status (total aerobic mesophilic bacteria, AM; total mesophilic anaerobic bacteria, ANM; lactic acid bacteria, LAB), pH, colour, lipid oxidation (acidic value, AV; peroxide value, POV; TBARs) and protein oxidation (fluorescence spectroscopy, λ_{ex-1} = 280-290 nm; λ_{ex-2} = 320-350 nm; λ_{ex-2} = 380-390 nm) were evaluated every 5-days along the 15 days of refrigerated storage (figure 9).

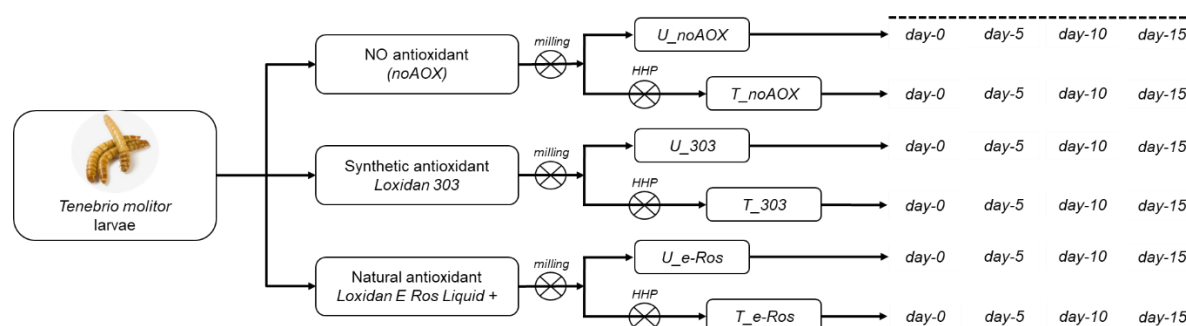


Figure 9. Graphic representation of the experiment 2.2. Adapted from Rossi *et al.* (submitted-A).

In both experiments, analysis of pastes before and immediately after the HHP treatment revealed that HHP was able to significantly ($P < 0.001$) reduce microbial load, with AM counts reduced of approximately 2-3 log, ANM reduced of 3-5 log, Y&M reduced of 4 log and LAB reduced of 5 log. Such results are consistent with previous studies (Rumpold *et al.*, 2014; Campbell *et al.*, 2020; Kashiri *et al.*, 2018; Larouche *et al.*, 2019) and confirm HHP as an efficient method for microbial load reduction. However, microbial reduction detected in experiment 2.2 was higher than in experiment 2.1 (3.40 vs 2.14 log reduction for AM; 4.60 vs 2.65 log reduction for ANM). On the other hand, no reduction of MS was detected with values of treated pastes being significantly higher ($P = 0.004$) than untreated pastes (3.19 ± 0.17 vs 3.91 ± 0.13). HHP is indeed known for not inactivate spore, although bacterial endospore inactivation has been observed when high temperature HHP was applied (Aganovic *et al.*, 2021). Interesting was to observe that presence of antioxidants in experiment 2.2 led to a lower initial microbial level, which translated into a lower microbial load detected after HHP treatment (figure 10B). It suggested an antimicrobial effect carried out by the antioxidant compounds as already noticed in

previous studies (Nieto *et al.*, 2018; Burel *et al.*, 2021; Eliuz, 2020). In both experiments, limited microbial growth was detected in HHP-treated pastes during the first 5 (experiment 2.2) and 7 (experiment 2.1) days of storage. Such limited growth was independent of the considered microbial parameter as well as by the applied packaging technology and by the presence of antioxidants. However, it was noticed that microbial growth was present in paste supplemented with antioxidants indicating a progressive reduction of the antimicrobial activity attributable to the antioxidant during the first 5 days of storage. Significant microbial contamination was indeed recorded in pastes supplemented with antioxidants after 10 days of storage, while contamination level of pastes without antioxidant was kept stable during this period (figure 10B). No effect of the packaging system was instead observed on microbial contamination level during the entire storage, with microbial contamination level stable for 7 days and quickly increasing from day-7 to day-14 (figure 10A). Absence of differences in microbial contamination level should however be considered as a consequence of the repackaging operated in experiment 2.1, which was instead avoided in experiment 2.2, where continuous vacuum was ensured along the entire experimental period. Taken together these results suggested that HHP may elongate the shelf life of insect paste to at least 10 days under refrigeration and vacuum packaging. Use of additives may further improve the microbial stability of the insect paste, although care should be paid on defining the right dosages.

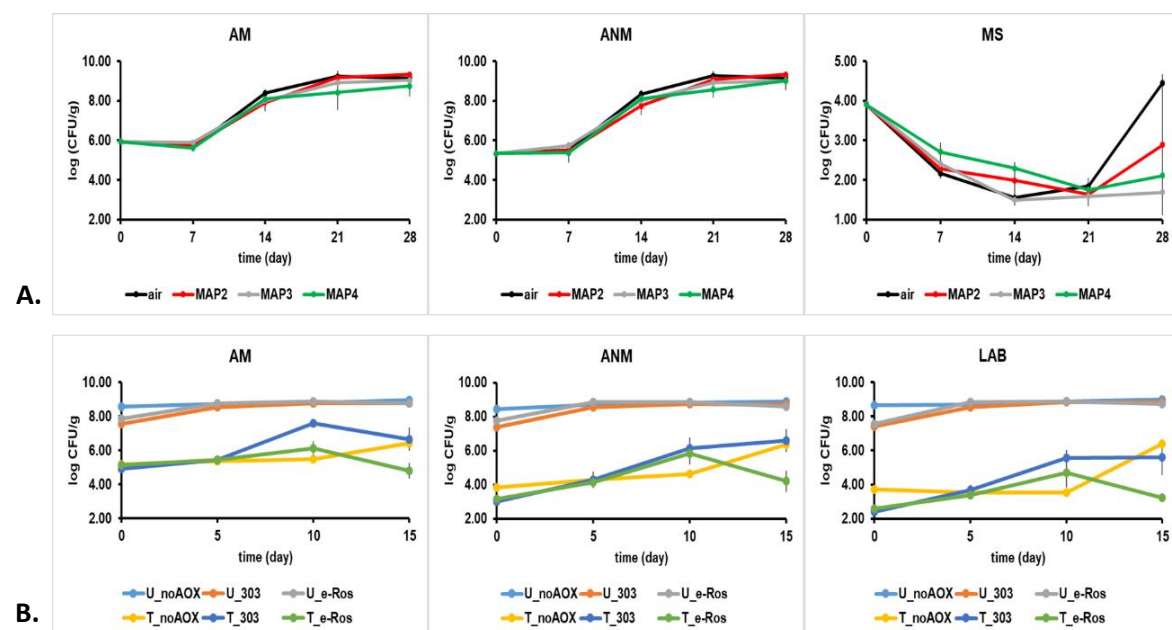


Figure 10. Microbial dynamics (AM, total aerobic mesophilic bacteria; ANM, total anaerobic mesophilic bacteria; MS, mesophilic bacteria spores; LAB, lactic acid bacteria) of *Tenebrio molitor* paste stored at 4°C under different MAP conditions (A; experiment 2.1) or supplemented with different antioxidants (B; experiment 2.2). Adapted from Ojha *et al.* (2023) and Rossi *et al.* (submitted-A).

While HHP reduced the microbial contamination of the food, several reports have highlighted that the same treatment can be responsible for promoting lipid oxidation (Alves *et al.*, 2012; Cropotova *et al.*, 2020; Medina-Meza *et al.*, 2014) as well as protein denaturation and oxidation (Tonnejck-Srpova *et al.*, 2019; Fuentes *et al.*, 2010). Nevertheless, contradictory results have often been reported, highlighting a matrix-dependent effect. Indeed, while pressures of at least 300 MPa are usually needed for lipid oxidation in meat (Medina-Meza *et al.*, 2014), higher lipid oxidation was recorded in mackerel fillets treated by HHP at 200 MPa rather than at 300 MPa (Cropotova *et al.*, 2020). On the other hand, clearer trend was usually observed for proteins, with higher pressures usually responsible for protein

aggregation and denaturation, which will result in lower solubility and higher oxidation (Cropotova *et al.*, 2020). Protein denaturation resulting in syneresis and serum release has indeed already been observed in *T. molitor* paste submitted to HHP at 400 and 500 MPa (Tonneijck-Sropa *et al.*, 2019). In the two considered experiments, lipid oxidation was investigated by measuring TBARs (experiment 2.1 and experiment 2.2), acidic value (AV) (experiment 2.2) and peroxide value (POV) (experiment 2.2). Investigations conducted before and immediately after the HHP treatment suggested that HHP at 600 MPa for 5 min was responsible for inducing lipid oxidation in mealworm pastes. Significantly higher TBARs were indeed computed in treated pastes from experiment 2.1 (TBARs in untreated pastes = 176.60 ± 7.07 $\mu\text{g MDA/Kg}$, TBARS in HHP treated pastes = 256.44 ± 7.07 $\mu\text{g MDA/Kg}$; $P = 0.0005$). On the other hand, the same effect was not detected in experiment 2.2 where lower TBARs were measured in treated pastes, rather than in untreated pastes (TBARs in untreated pastes = 277.45 ± 3.94 $\mu\text{g MDA/Kg}$, TBARS in HHP treated pastes = 254.65 ± 2.00 $\mu\text{g MDA/Kg}$). However, it should be noted that, while the differences observed in experiment 2.2 were not significant, the TBARs value of initial pastes in this experiment was significantly higher than the TBARs value of the initial pastes in experiment 2.1. This difference, which might be associated with the different preparation method applied to the pastes from the two experiments (frozen larvae were directly milled in experiment 2.1, while they were defrosted overnight before milling in experiment 2.2), may suggest an innate antioxidant activity of *T. molitor* larvae, which might therefore compensate the pro-oxidant effect advocated to HHP. Similarly, POV (accounting for primary products of the oxidation process) and AV (accounting for the amount of free fatty acids present within the paste) measured in experiment 2.2 decreased after the HHP treatment. This effect might suggest a progressive inactivation of the lipases responsible for hydrolysis of the glycerides on one hand, while on the other hand, a stabilisation effect of the peroxides, which might be degraded during the HHP treatment. Moreover, no noteworthy effect was explicated by the antioxidants during the treatment, making their application unnecessary. Considering the evolution of lipid oxidation products during the storage, experiment 2.1 suggested that the removal of oxygen in direct contact with the paste could reduce the aldehydes production. Pastes stored in MAP-4 indeed showed significantly lower TBARs than the other pastes after 28 days of refrigerated storage (figure 11A). Lower TBARs were also detected in pastes stored in MAP-2 during the first 14 days of storage, although such differences disappeared during the following days. The application of antioxidants on the pastes did not appear to carry any positive effect on HHP-treated paste during the storage (figure 11B). TBARs values of treated pastes supplemented with antioxidants indeed tend to progressively increase during the storage, reaching levels significantly higher than treated pastes without antioxidants. A more marked effect attributable to antioxidants was instead observed for untreated pastes, which showed lower TBARs after 15 days of refrigerated storage whether antioxidant mixtures were applied. A clearer antioxidant effect was instead detected in terms of POV. Indeed, while stable peroxide value was computed for treated and untreated pastes without antioxidants, a progressive decrease of POV was measured in treated and untreated pastes with antioxidants. However, while the activity of the artificial antioxidant was mainly exercised during the first 10 days of storage, natural antioxidants showed higher stabilisation activity during the last five days of storage, suggesting a different mechanism of action of the two commercial antioxidant blends.

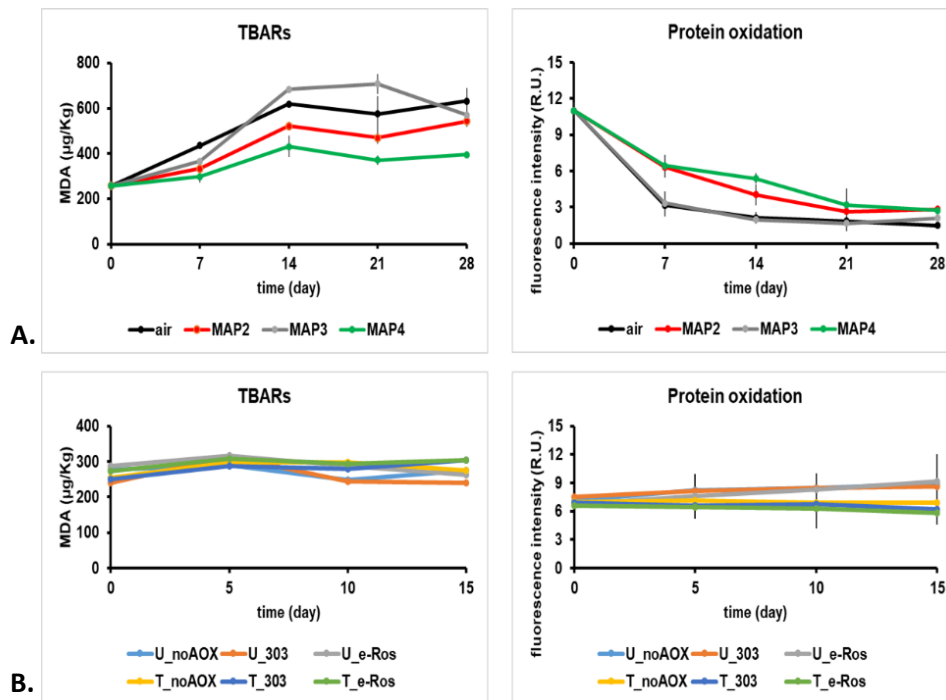


Figure 11. Lipid oxidation (TBARs, μg malondialdehyde (MDA)/Kg paste) and protein oxidation (fluorescence intensity, $\lambda_{ex} = 285$ nm) of *Tenebrio molitor* paste stored at 4°C under different MAP conditions (A; experiment 2.1) or supplemented with different antioxidants (B; experiment 2.2).

Adapted from Ojha *et al.* (2023) and Rossi *et al.* (submitted-A).

Protein oxidation was determined by applying fluorescence. Emission spectra recorded in the excitation regions 250-290 nm (experiment 2.1) and 280-290 nm, 320-350 nm, 380-390 nm (experiment 2.2) were resolved through PARAFAC analysis and evolution of maximum fluorescence intensity recorded at the PARAFAC extracted peaks was evaluated during the storage period. PARAFAC carried out on the spectra recorded within the experiment 2.1 showed the presence of two main peaks located at excitation wavelengths 280 nm and 285 nm, and both ascribable to tryptophan. Specifically, while the peak at 280 nm described the protein unfolding with the tryptophan molecules going to be exposed on the external structure of the protein, the emission peak at 285 nm showed a progressive decreasing trend during the storage. Such trend closely followed the increasing trend described by the TBARs, with values recorded from oxygen free packaging (MAP-2 and MAP-4) being higher and well separated from the values recorded from the samples packed in presence of oxygen (air and MAP-3) (figure 11A). Regression analysis carried out between TBARs and fluorescence intensity showed that a high correlation was detected ($R^2 = 0.7802$, $P < 0.001$), suggesting that fluorescence intensity recorded at excitation 285 nm could closely describe the protein oxidation happening within the paste. The same two fluorescence peaks detected in experiment 2.1 were also observed in experiment 2.2. However, while the peak at 280 nm was again able to detect the redshift due to HHP, no clear trend was observable for the maximum fluorescence intensity associated with the peak at 285 nm (figure 11B), although a slight increase of such peak was observed during the storage in untreated pastes. Overall, these two studies suggested that *T. molitor* pastes are particularly prone to oxidation, which can be triggered by the application of HHP. Addition of antioxidants to the initial paste seems not to carry any protective effect, although it might allow high oxidation stability whether long storage periods are applied. In such a case, application of vacuum packaging or MAP system without oxygen should however be preferred, although due to microbial stability, storage of HHP-treated mealworm pastes should never exceed 10 days under refrigerated conditions.

3.3 *Insects as a novel tool for aquaculture waste management*

When food is produced, a significant amount of organic waste is generated. Focusing on the only production stage, which includes plant growth, animal breeding and primary processing, approximately 1 billion of tons of waste are produced yearly (Obi *et al.*, 2016). Such waste, which can overall be defined as agricultural waste, can be further categorised in crop residues (e.g. straw and leaves), industrial processing waste (e.g. fruit peels, egg shells, rice bran, rice husk, bagasse, molasses), livestock waste (including manure) and fruit waste (residuals from harvest) (Awogheni and Von Kallon, 2022).

Global production of livestock (domesticated animals raised for producing meat, eggs, milk, fur, leather and wool or for providing labour) manure accounts for approximately 3.12 billion of tons every year (FAIRR, 2022). Such amount is however expected to be underestimated as it only considers manure produced within barns (not from pasture animals) and only includes a limited range of animal species (cattle, swine, poultry and horses). Fish is therefore not included.

Fish production is the fastest-growing food sector, as well as the most important one in the economy of several developing countries (Garlock *et al.*, 2022). Recent UN estimates have highlighted that in 2022, approximately 222.3 million of metric tons of fish and seafood were produced globally (FAO, 2024). Of this amount, more than 50% originates from aquaculture, which in 2018 was responsible for providing about 82.1 million metric tons of aquatic animals and 32.4 million metric tons of algae (FAO, 2022b). Such amounts increased in 2022, with 130.9 million metric tons of fish and seafood originating from aquaculture (FAO, 2024). Considering that in 2023 the amount of animal meat (including pork, beef, poultry, sheep and goat) produced globally was approximately 350 million metric tons (150 million = poultry, 100 million = pork, 50 million = beef and veal, 20 million = sheep and goat, 30 million = others) (www.statista.com), it is possible to understand how fish production plays a crucial role in human nutrition. It is indeed estimated that approximately 10% of the total protein and 15% of the animal protein consumed globally originates from fish and seafood, with some African and Asian countries, where these amounts rise to more than 50% (Tidwell and Allan, 2001).

One of the main problem connected with aquaculture production is represented by the generation of waste (Henares *et al.*, 2020). Aquaculture waste are represented by fish carcasses, uneaten feed, faecal drops and metabolic products (Amirkolaie, 2011), and, being rich in nitrogen, phosphorous and organic matter (Dauda *et al.*, 2019), might be responsible for eutrophication, production of GHG and reduction of the fish rearing performances (Drozd *et al.*, 2020; MacLeod *et al.*, 2020). Management of such waste is traditionally carried out through landfilling, incineration, wetland, phytoremediation, composting, aerobic/anaerobic digestion and direct fertilisation (Wu and Song, 2021; Ahmad *et al.*, 2022). Alternative and more modern methods also include aquaponics, biofloc and vermicomposting (Klein *et al.*, 2023; Wu and Song, 2021). The idea of using aquaculture waste for rearing insects has only been developed recently (Schmitt *et al.*, 2019). Specifically, in the study carried out by Schmitt *et al.* (2019), oven-dried aquaculture waste from salmon production was reconstituted at 70% of moisture and directly fed to BSFL. Although insects appeared to be able to grow on such waste, growth rates were extremely low, with final weight amounting for approximately 60-70 mg/larvae. Additionally, heavy metals present within the waste tended to accumulate within the larvae, raising a safety concern whether such insects were applied as animal feed (Schmitt *et al.*, 2019). In a follow-up study, Liland *et al.* (2023) confirmed such findings but observed that larval growth could significantly improve if commercial chicken feed was added to the waste.

Therefore, BSFL can grow on waste from aquaculture production. However, both studies focused on insect production, neglecting the waste conversion aspect. Additionally, both studies used dried waste. Drying operations, besides being energy-demanding, can be responsible for vitamins and other chemical compounds degradation, as well as for microbial load reduction. Such processes might therefore have significantly reduced chemical and microbial quality of the substrate resulting in lower BSFL growth performances (Oonincx *et al.*, 2015; Isibika *et al.*, 2023).

In this section, results of two researches (annex 8, Rossi *et al.* (2023a); annex 9, Rossi *et al.* (submitted-B)) are presented and discussed. The aim of these researches was to explore the ability of BSFL to convert fresh ASW originated from recirculating aquaculture systems (RAS, figure 12). RAS can be defined as a closed fish rearing system where the rearing water is continually recycled through a system of filtration, purification and disinfection (Henares *et al.*, 2020). The aim of such system is to minimise the environmental impact of the aquaculture production by reducing water use: fish rearing water exiting the rearing pond is indeed subjected to solid removal, biofiltration, CO₂ removal, disinfection, pH adjustment and oxygenation and reintroduced within the pond (Ebeling and Timmons, 2012).

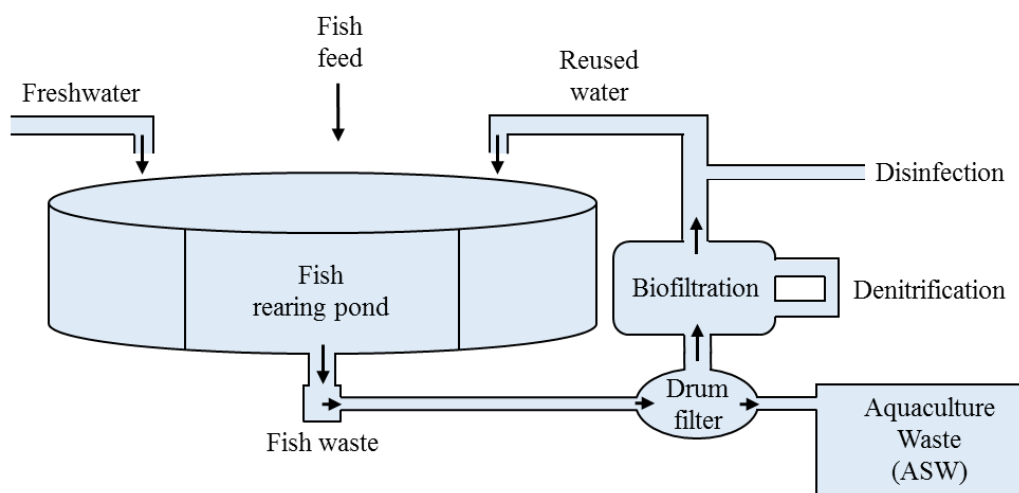


Figure 12. Schematic representation of recirculating aquaculture system (RAS) originating the aquaculture waste used in the two presented experiments. Adapted from Rossi *et al.* (submitted-B).

Within the experiment 3.1 (annex 8, Rossi *et al.*, (2023a)), aquaculture waste from pikeperch (*Sander lucioperca*) reared at the Institute of Inland Fisheries (Potsdam-Sacrow, Germany) was used. Wastewater (dry matter = 0.02%) originating from drum filter was collected daily over 2-weeks period and submitted to a concentration process consisting of sedimentation, filtration and centrifugation steps. Resulting material (dry matter = 9.07%) was then stored at -18°C until the start of the experiment.

In the experiment 3.2, aquaculture solid waste (ASW) were provided by an industrial fish rearing facility located at the Blankensee lake (Trebbin, Germany) and rearing pikeperch (*Sander lucioperca*) and European catfish (*Silurus glanis*). Within the rearing facility, wastewater containing solid particles was collected every second day, dewatered, conveyed to a sedimentation pond and subjected to an anaerobic digestion process. Collected waste was therefore concentrated through sedimentation and filtration and residual material (dry matter = 25.01%) was stored at -18°C until the start of the experiment.

In both experiments, four rearing substrates were prepared by mixing defrosted ASW with chicken feed in different percentages. A fifth substrate, consisting of pure chicken feed was further prepared and used as control (table 10). If needed, distilled water was added to achieve a final moisture content of the substrate of 70%, considered to be optimal for BSFL growth (Bekker *et al.*, 2021). For each treatment, three experimental replicates were prepared by adding 300 g of fresh substrate and 200 BSFL (5-days old) in each rearing box, ensuring a rearing density of 0.6 larvae/cm² and a feeding rate of 1.5 g of fresh feed/larvae (i.e. 0.45 g of dry feed/larvae if substrate moisture was 70%). Such feeding rate, although not optimal, appeared to satisfy the minimum requirements defined by Diener *et al.* (2009), according with whom, 100 mg feed (60% of moisture)/larvae/day (i.e. 1 g feed/larvae if 10 days of rearing period are considered) are an optimum trade-off between material reduction and larval

biomass production. Batch feeding system was applied as it was considered the best performing feeding strategy when substrate reduction is the goal (Dzepe *et al.*, 2021). However, as ASW used in experiment 3.1 had a much lower dry matter, the adjusted feeding rate on dry matter bases in substrate 100ASW was only 0.14 g of dry feed/larvae. Feeding rate of 0.45 g of dry feed/larvae was instead ensured in experiment 3.2 by adding a surplus of substrate in the treatment 100ASW.

Table 10. Composition of the experimental substrates used in the two presented experiments. Adapted from Rossi *et al.* (2023a) and Rossi *et al.* (submitted-B).

Substrate	Aquaculture solid waste (%)	Chicken feed (%)
0ASW (control)	0	100
25ASW	25	75
50ASW	50	50
75ASW	75	25
100ASW	100	0

BSFL were then let grow until the first prepupae, applying 27°C of temperature, 70% of relative humidity (RH) and 0:24 (L:D) of photoperiod (Meneguz *et al.*, 2018). Larval growth was monitored along the overall rearing period by randomly sampling and weighing 10 larvae/box. Rearing performances and waste bioconversion performances were also considered by computing several parameters (survival, development time, gained weight per larvae, assimilated feed, feed conversion ratio, biomass conversion rate and substrate reduction), as suggested by Surendra *et al.* (2020) and Bosch *et al.* (2020).

Results of the two experiments shown that ASW can be converted by BSFL. However, while fresh ASW applied in the experiment 3.1 can theoretically be used as it is, anaerobically digested ASW does not allow larval growth (figure 13).

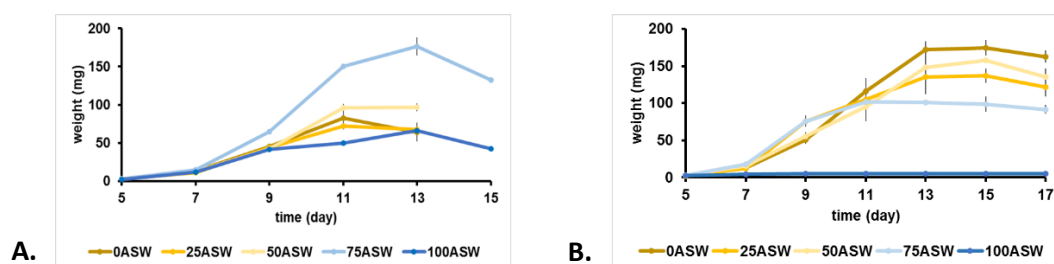


Figure 13. BSFL growth on the different experimental substrates from experiment 3.1 (A) and experiment 3.2 (B). Adapted from Rossi *et al.* (2023a) and Rossi *et al.* (submitted-B).

The main motivation for such differences in growth was attributed to the different chemical compositions of the two ASW used in the experiments and resulting from the anaerobic digestion that happened in experiment 3.2, but not in experiment 3.1. Indeed, although both fish species are carnivorous, whereby they fed on diets rich in protein and poor in carbohydrates, the chemical composition of the two ASW was extremely different, with the ASW used in experiment 3.2 being higher in ash (~60% dry matter bases) and lower in lipids (~0.40% dry matter bases) than ASW used in experiment 3.1 (figure 14). While lipids have been suggested as an energy source, whereby their presence is responsible for biomass accumulation and higher larval weight (Manurung *et al.*, 2016), ash, which is a non-energetic component of the diet, is inversely correlated with final larval weight (Oddon *et al.*, 2024; Lalander *et al.*, 2019), suggesting that no larval growth could be expected in diet 100ASW from experiment 3.2. Addition of chicken feed to the ASW led to a reduction in ash and an increase in lipids and non-fibrous carbohydrates suggesting better larval growth. Additionally, substrates consisting of mixtures of ASW and chicken feed appeared to be more chemically balanced, suggesting better substrate utilisation by the larvae and higher larval growth (Cammack and Tomberlin, 2017; Barragan-Fonseca *et al.*, 2018; Lopes *et al.*, 2020; Gold *et al.*, 2020).

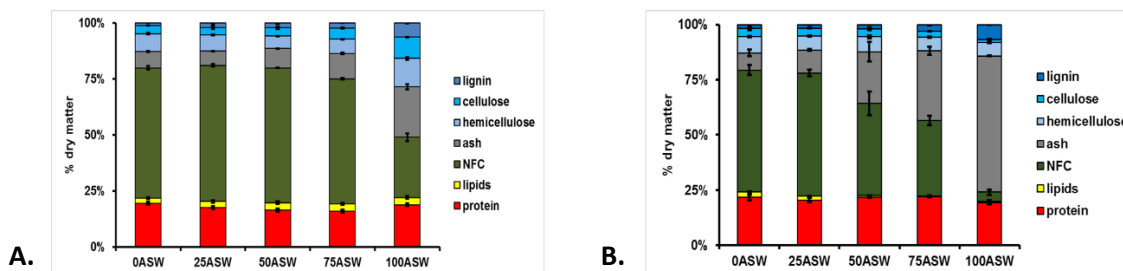


Figure 14. Chemical composition of experimental substrates applied in experiment 3.1 (A) and experiment 3.2 (B). Adapted from Rossi *et al.* (2023a) and Rossi *et al.* (submitted-B).

However, although a linear regression could be drawn between amount of ASW in diet and final larval weight for experiment 3.2 ($R^2 = 0.9301$, $P = 4.272e-09$), it was not the case for experiment 3.1, where the substrate 75ASW yielded better insect growth than all the other substrates. Suitable explanation for the low growth observed in the treatments 0ASW, 25ASW and 50ASW of experiment 3.1 might be the presence of a water layer on the surface of the substrates. Significant amount of extra water was indeed added in these treatments and its presence might have been resulted in lower oxygen dissolution within the substrate, suggesting difficulties for larvae to breathe and grow (Frooninckx *et al.*, 2024). Additionally, such water evaporated fast, leaving a dry substrate not consumable by the larvae (Bekker *et al.*, 2021). Higher levels of ASW in diet could prevent such excess of free water, therefore leading to better larval performances and higher growth. The low final weight reached in the treatment 100ASW of experiment 3.1 could instead be explained by the low feeding rate on dry matter bases applied in such treatment, which resulted in a low amount of nutrients and energy for sustaining larval growth (Diener *et al.*, 2009; Manurung *et al.*, 2016). However, as the larval survival on this substrate was not significantly different than the other substrates, the amount of feed could have been considered satisfactory for sustaining larval development and promoting pupation. BSFL are indeed characterised by a compensatory mechanism (Barragan-Fonseca *et al.*, 2018), whereby they are able to change their behaviour as response to different external conditions. Specifically, in the examined case, they consumed the substrate (especially proteins) in a way that allowed them to reach the critical weight (minimum weight required for pupation), prioritising development to the energy accumulation (Pimentel *et al.*, 2017) and resulting in larvae ready for pupation but with low final weight (Koyama *et al.*, 2018).

Such a condition might also have played a positive role in terms of feed consumption and substrate utilisation. Indeed, nutrients consumption by the insects strictly depends on regulatory mechanisms happening within the larval fat body. Specifically, while the insects tend to seize nitrogen and accumulate proteins during the first stage of the development, they prioritise lipid assimilation and carbon accumulation during the second stage (fattening) (Barragan Fonseca *et al.*, 2018). It is translated in a not balanced nutrients utilisation, which will lead to lower nutrients retention and higher volatilisation. Mass balance analyses carried out in experiment 3.1 indeed showed that only 23% of the total N and 22% of the total C was lost by volatilisation in treatment 100ASW, while significantly higher losses were detected in treatment 75ASW, which however resulted in higher larval biomass production (figure 15a).

Mass balance analyses carried out in experiment 3.2 (figure 15b) further confirmed such observation, while the amounts of nitrogen and carbon lost by volatilisation amounting to approximately 70%. No feed limitation was indeed applied on this experiment, where all the substrates (except substrate 100ASW) allowed to reach satisfactory larval weight.

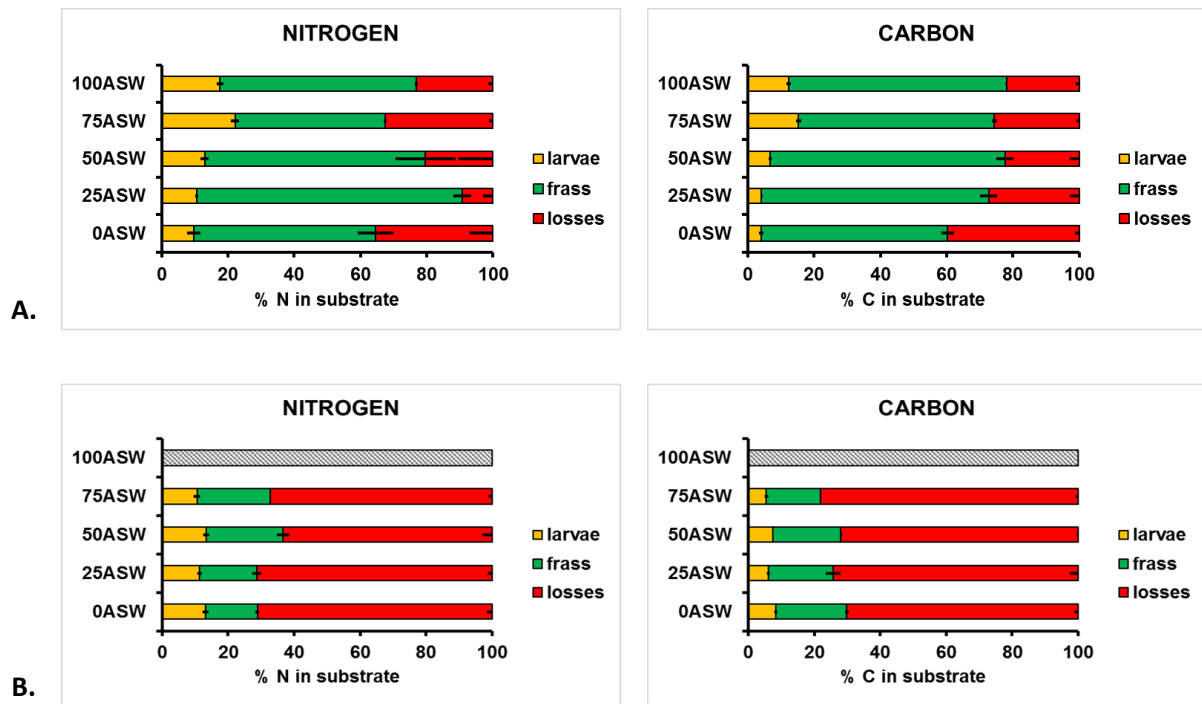


Figure 15. Mass balance analyses of Nitrogen and Carbon recorded on the different experimental substrates from experiment 3.1 (A) and experiment 3.2 (B). Adapted from Rossi *et al.* (2023a) and Rossi *et al.* (submitted-B).

Interesting observation was also that, although nutrients retention was better when feed limitation was applied, the same condition did not lead to better substrate utilisation. Amount of assimilated feed, substrate reduction and bioconversion ratio (i.e. the amount of substrate converted in insect mass) computed for substrate 100ASW in experiment 3.1 were indeed significantly lower than for the other substrates (table 11), suggesting that when the waste conversion is the objective of the process, optimisation based on larval biomass production should not be carried out. Similarly, a low feed conversion ratio (i.e. the amount of feed needed for obtain the increase of one gram of larval weight) as the one observed for treatments 75ASW and 50ASW in experiment 3.1 or for treatments 50ASW and 0ASW in experiment 3.2, although typically synonym of a good substrate utilisation, might not always be associated with an acceptable nutrient assimilation and low environmental impact of the process. Insect-mediated waste conversion process is indeed a complex operation, where a wide range of parameters, including pH, protein/carbohydrates ratio, C/N ratio, fibre levels and composition, etc., play a role (Cammack and Tomberlin, 2017; Pinotti and Ottobon, 2021; Rehman *et al.*, 2017a, 2017b). Such conditions, besides directly affecting larval performances, may also influence microbial growth. Microorganisms are crucial for degrading indigestible dietary components (Rehman *et al.*, 2017a), making the overall substrate easier to exploit by the larval and supporting larval survival and development on one hand, while promoting substrate utilisation and waste conversion on the other hand (Gold *et al.*, 2018; Manna *et al.*, 2024).

Table 11. Bioconversion performances of BSFL computed on the different experimental substrates from the two studied experiments. Adapted from Rossi *et al.* (2023a) and Rossi *et al.* (submitted-B).

Experiment	Substrate	Final larval weight (mg)	Assimilated feed (g)	Feed conversion ratio	Bioconversion ratio (%)	Substrate reduction (%)
Experiment 3.1	0ASW	75.41 ± 9.38	47.64 ± 2.42	14.08 ± 2.69	4.04 ± 0.72	43.43 ± 2.10
	25ASW	67.61 ± 4.62	25.49 ± 2.68	9.18 ± 1.84	3.23 ± 0.30	28.07 ± 2.92
	50ASW	85.57 ± 7.04	21.22 ± 2.44	4.55 ± 0.89	4.89 ± 0.44	23.88 ± 2.79
	75ASW	176.30 ± 12.12	32.14 ± 1.75	2.80 ± 0.08	9.68 ± 0.52	35.68 ± 1.14
	100ASW	66.17 ± 1.05	5.28 ± 0.07	1.78 ± 0.08	3.92 ± 0.20	22.11 ± 0.16
Experiment 3.2	0ASW	162.65 ± 4.81	42.35 ± 2.17	5.74 ± 0.75	6.89 ± 0.39	42.97 ± 1.26

	25ASW	121.82 ± 7.59	48.92 ± 13.91	8.82 ± 1.94	5.07 ± 0.33	43.95 ± 7.39
	50ASW	135.38 ± 6.53	31.23 ± 1.32	5.66 ± 0.25	5.78 ± 0.01	35.27 ± 1.17
	75ASW	91.48 ± 3.38	37.46 ± 0.55	13.11 ± 0.38	4.34 ± 0.20	42.08 ± 0.51
	100ASW	--	--	--	--	--

3.4 Quantification of greenhouse gas emission during edible insect rearing

One of the main reason supporting the use of edible insects as future food and feed source is related to the low environmental impact associated to their production. Recent estimates suggest that the current traditional agriculture is responsible for approximately 20% of the overall GHG emissions (Duque-Acevedo *et al.*, 2020). Of this amount, the agricultural activities related to the only food production (crops growing and livestock rearing) account for approximately 7.4 Gt of CO₂-eq per year (14% of the total global GHG emission) (FAO, 2022c), which increases to 15.8 Gt CO₂-eq if transport, processing, storage, distribution and consumption of food are considered (Li *et al.*, 2022). A wide range of these GHG emissions (~ 30% of the overall food supply chain emission) is shared among livestock and fish production (Poore and Nemecek, 2018) and the only enteric fermentation is considered to be responsible for approximately 40% of the total GHG emissions recorded at farm level (~ 2.8 Gt CO₂-eq) (FAO, 2022c).

Different animal species are responsible for different GHG production, with cattle, sheep and pig producing approximately 35, 24 and 7 Kg CO₂-eq/Kg of meat, respectively, and aquaculture and poultry releasing 9.7 and 6 Kg CO₂-eq/Kg of meat, respectively (FAO, 2022c; Smetana *et al.*, 2023). It is therefore evident that reducing meat consumption as well as shifting from ruminant meat to poultry meat can significantly reduce the environmental impact associated with the food system (Mazac *et al.*, 2022; van Huis and Oonincx, 2017). Insects are usually associated with low GHG emissions, with black soldier fly larvae showing approximately 2.3-3.1 Kg CO₂-eq/Kg (Bosch *et al.*, 2019; Bava *et al.*, 2019), mealworms 2.7 Kg CO₂-eq/Kg (Oonincx *et al.*, 2012) and crickets 2.3-2.6 Kg CO₂-eq/Kg (Halloran *et al.*, 2017).

GHG emissions of edible insects are however strictly affected by different rearing conditions, including rearing substrate, dietary quality (nutrients, C/N, pH, moisture content), rearing density, feeding rate, feeding strategy (batch vs continuous), air temperature, air humidity, ventilation rate, light intensity, insect age and rearing duration (Smetana *et al.*, 2023; Boakye-Yiadom *et al.*, 2022; Pang *et al.*, 2020a, 2020b; Chen *et al.*, 2019).

The device and the sampling procedure and frequency applied for measuring GHG emission might also significantly impact the GHG quantification (Parodi *et al.*, 2020), with closed static chambers being the most used but also the less precise ones and the open system being precise but costly and difficult to operate. Closed static chambers as described by Mertenat *et al.* (2019) and Chen *et al.* (2019) consist in simple containers where the insects are hosted for the entire life cycle or for a specific time interval. At the moment of gas measurement, these containers are hermetically closed, allowing an increasing of the gas concentration in the air within the chamber. Such air is therefore discontinuously sampled and gas concentration is determined. The evolution of such a system is represented by the closed dynamic chamber as described by Guo *et al.* (2021), where the gas concentration in the air within the chamber is continuously measured for a limited period of time, ranging from a few hours to a few days. The main problem connected with these systems is related to a continuous increasing of exhausted gasses and decreased of fresh clean air inside the chamber. Air rich in humidity and CO₂ and poor in O₂ is therefore the only available for the insects during the measuring time. Such conditions, which are not commonly encountered in an insect production facility, might strongly affect the metabolic performance of the insects, resulting in significantly different GHG emissions (Akbar *et al.*, 2016; Mertenat *et al.*, 2019). Dynamic open chambers are instead systems that continuously pump ambient air through the chamber allowing it to closely simulate the real insect rearing conditions (Parodi *et al.*, 2020). However, despite this system appears for being more accurate and precise than closed systems,

it usually requires high amount of substrate and insects, therefore being difficult to operate and control during long periods. New systems for fast, accurate, cheap and reliable quantification of GHG emissions from edible insects are therefore needed.

This section aims to present and discuss the results of a recent research article (annex 10, Rossi *et al.* (2024b)) where a new small-scale open chamber is described and tested. The developed device consisted in four independent small open chambers (figure 16), each one characterised by an inlet and outlet system, enabling a continuous airflow through the chamber. It allowed the insects to continuously have fresh air (high in oxygen and low in CO₂) available for growing and carrying out their metabolic activities, therefore strictly simulating the actual insect rearing conditions. According to the chamber layout, temperature and humidity conditioned air was generated by an industrial climate chamber (a) and conveyed into a central input box (b) for sampling. Fresh air was therefore pumped within each single small open chamber (c) hosting substrate and insects. The overpressure generated within every chamber was therefore responsible for flowing the exhausted air through the outlet line (red pipes in figure 16) which terminated with a flow meter (d) responsible for air flow detection. Each outlet line was further provided with a septum for gas sampling (e).

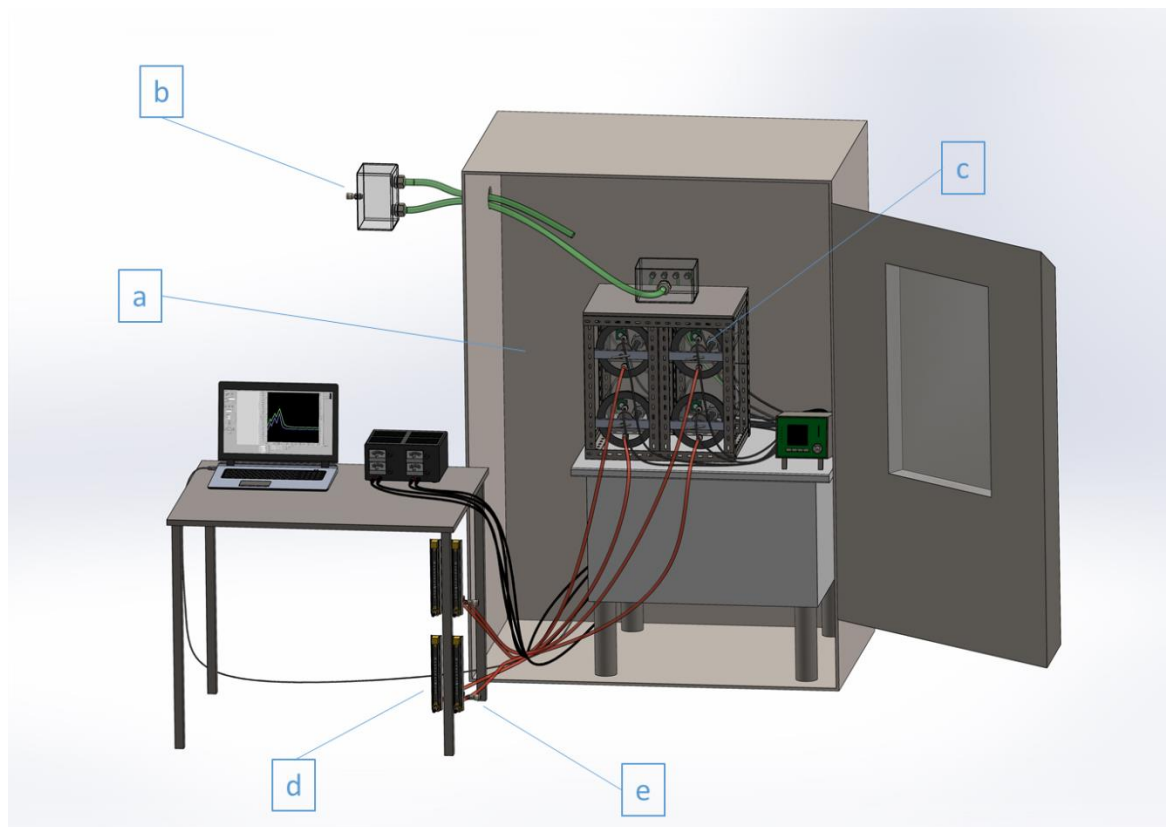


Figure 16. Overview of the gas emission chamber for recording greenhouse gas emission from edible insects. Adapted from Rossi *et al.* (2024b).

Built device was tested by studying the GHG emissions of BSFL reared on commercial chicken feed. For this purpose, four substrates containing chicken feed moistened with distilled water at 60% of final moisture (final substrate weight = 300 g) were prepared. Exactly 200-less than 12 hours old BSFL were seeded in two of the four substrates (feeding rate = 1.5 g of fresh substrate/larvae or 0.6 g of dry substrate/larvae) and the box containing solo substrate (treatment C) or substrate with larvae (treatment T) were placed inside the chambers. Gas sampling was carried out by syringing inlet and outlet air every day at 8:00 am and 8:00 pm along 12 days of rearing period. Every sampling consisted of three replications, which results were averaged. Following the sampling, CO₂, N₂O and CH₄

concentrations within the inlet and outlet air were quantified by gas-chromatography. Three independent replicates (run) with two replicates/run were conducted. Gas emission curves were built by considering a linear tendency between two successive sampling points and cumulative gas emissions were determined by applying the area-under-the-curve procedure. Intra and inter run reproducibility were determined by multiple cross-correlation analysis. Results showed that cross-correlation analysis related to CO₂ emission gave a correlation coefficient > 0.9 while weaker correlations were computed for CH₄ ($r = 0.15$) and N₂O ($r = 0.16$) which could however be due to the low amounts of these gasses recorded in the outlet air. Such observations, which confirmed previous results (Lindberg *et al.*, 2022), according to which BSFL are not directly able to produce any N₂O or CH₄, suggest that replacing traditional animal protein sources with edible insects might contribute to a significant reduction of the environmental impact associated to the current food system. Emissions of N₂O and CH₄ in edible insects rearing are indeed generally associated to chemical as well as microbiological processes happening within the substrate. Mertenat *et al.* (2019) for example observed that N₂O emissions were mainly located just after the feeding event, i.e. when the pH of the substrate was still low and the nitrate concentration was high. N₂O emissions are indeed due to a denitrification process happening within the substrate during the first hours of composting, i.e. when anaerobic conditions are still present and denitrification bacteria are free to operate (Pang *et al.*, 2020b). Similarly, CH₄, being associated with methanogenic bacteria (Chen *et al.*, 2019), has only been detected when waste or manure were used (Parodi *et al.*, 2021). No methanogenic bacteria have indeed been identified in BSFL gut so far (Jeon *et al.*, 2011) and their presence in substrate appears to be strictly connected to the anaerobic conditions (Boakye-Yiadom *et al.*, 2022). As in the described study, chicken feed provided through a single feeding event was used, no significant N₂O and/or CH₄ production could be expected.

Further analysis conducted on the CO₂ emission pattern (figure 17) suggested that CO₂ emissions strictly followed the insect growth process. Belt-shaped emission curves, characterised by low CO₂ recovery during the first 3.5 days, followed by an increase between day 4 and day 7 and a quick decrease between day 7 and day 9, were indeed observed in all the runs. It suggests that CO₂ production is mainly due to insect metabolic activity on one hand, while on the other hand, it indicates that higher insect growth results in higher CO₂ emission. A strict relation between CO₂ emission and final larval weight has already been observed by Laganaro *et al.* (2012), further supporting the idea explained in the section 3.4, according to which optimising an insect-mediated waste conversion process by only considering the biomass production aspect might result in negative environmental consequences.

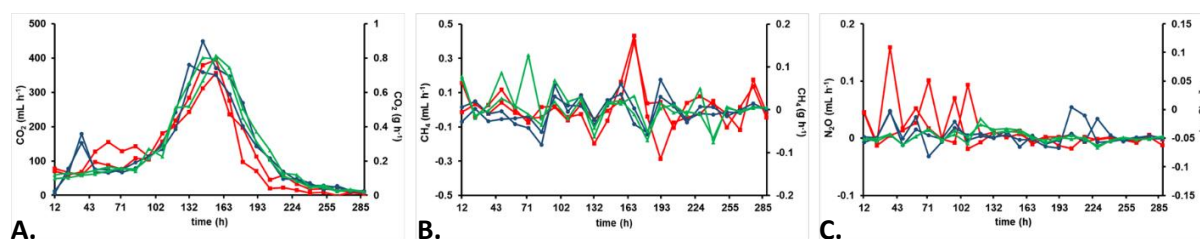


Figure 17. Gas emission curves for CO₂ (A), CH₄ (B), N₂O (C) detected from BSFL reared on moistened chicken feed from run-1 (red curves), run-2 (blue curves) and run-3 (green curves). Adapted from Rossi *et al.* (2024b).

Additionally, the fact that CO₂ emissions followed insect growth and weight accumulation suggests that the young larvae, which are metabolically very active (Gligorescu *et al.*, 2019), can be considered responsible for a significant amount of GHG production. It leads to the conclusion that in order to correctly quantify the GHG emissions associated with insect rearing, the overall insect life cycle from

eggs to adults should be considered. Cumulative CO₂ emissions detected in this study (table 12) were indeed significantly higher than the already published values (Boakye-Yiadam *et al.*, 2022).

Table 12. Cumulative gas emission detected from BSFL reared on moistened chicken feed along the three experimental runs (experimental) and normalised for gram of dry insect biomass (g-gas/g-insect (DM)). Adapted from Rossi *et al.* (2024b).

	CO ₂		CH ₄		N ₂ O	
	Experimental (g)	g-gas/g-insect (DM)	Experimental (mg)	mg-gas/g-insect (DM)	Experimental (mg)	mg-gas/g-insect (DM)
RUN-1	64.34 ± 3.12	9.36 ± 0.44	7.62 ± 1.14	1.3 ± 0.27	9.10 ± 0.12	1.33 ± 0.11
RUN-2	73.75 ± 1.38	11.88 ± 3.79	4.41 ± 0.52	0.74 ± 0.32	4.99 ± 0.29	0.79 ± 0.23
RUN-3	72.88 ± 0.27	7.76 ± 1.14	2.62 ± 0.21	0.28 ± 0.02	7.45 ± 2.13	0.76 ± 0.11

However, despite the highest accuracy reached in our study where 12 hours old larvae were used, such data might still not perfectly simulate the real CO₂ emission of BSFL reared on chicken feed. Some experimental constraints should indeed be considered. The first constraint is represented by the applied feeding system. Specifically, as we applied a batch feeding procedure, fresh feed was provided to the larvae only at the beginning of the experiment. It means that larvae consumed feed with high efficiency at the beginning of their life, while no easy-to-consume feed was available during the fattening stage. As BSFL rearing is usually organised in at least two feeding events (Deruytter *et al.*, 2023), with the first one happening on the neonates (starter feed) and the second applied on 5 to 7 days old larvae, the real expected CO₂ emission might be even higher than detected by us (Zhang *et al.*, 2021). The second constraint concerns the stage when the experiment was interrupted. BSFL were indeed harvested after 12 days of rearing. The harvesting period was defined based on the reduction of CO₂ production rate. However, at this stage the larvae still appeared to be quite away from turning into prepupae, which is the stage where BSFL are usually harvested for food or feed purposes (Deruytter *et al.*, 2023). Interrupting the gas measuring at this early time might be translated in significantly lower CO₂ emissions, which should be accounted in further studies. A third constraint is finally represented by the applied feeding rate. In this study, 200 BSFL were seeded into 300 g of feed. Although these proportions followed the recommendations from previous studies (Diener *et al.*, 2009), they appear to be different from the conditions applied in industrial production, where significantly lower feeding rates are applied for neonates larvae (Deruytter *et al.*, 2023). As different feeding rates are known to significantly affect BSFL growth and waste conversion (Chen *et al.*, 2019; Paz *et al.*, 2015), parameters such as larval density and feeding rate should carefully be considered when data comparison is performed.

4. Conclusions and outlook

Insects have been identified as a promising tool for promoting a transition from a traditional linear economy to a modern circular bioeconomy, while promoting continuous economic growth. Their ability to grow on underutilised substrates, recover otherwise lost nutrients and convert them into novel high-quality ingredients to be used in the agricultural, medical, veterinary, environmental and industrial sectors is already well known. However, although these concepts are largely accepted by the scientific community, several questions remain unanswered, preventing the full exploitation of the insect potential in daily life. Therefore, this thesis aimed to fill a part of such gaps, widening the knowledge on the use of insects in the modern economy and providing information regarding the application of environmentally-friendly insect-based waste conversion tools.

Starting with the four research questions outlined at the beginning of this thesis (chapter 1.2), the six experiments described in chapter 3 allowed to develop the following conclusions:

- (i) HHP combined with oxygen-free MAP or vacuum packaging can be considered as a suitable and “non-invasive” strategy for shelf life elongation of edible insect products, guaranteeing a significant reduction of microbial load while preserving chemical, nutritional and organoleptic quality of the final product. When combined with refrigerated storage, these technologies are able to guarantee at least 10 days of shelf life for fresh insect pastes;
- (ii) BSFL can conveniently grow on fresh, daily-collected ASW, configuring themselves as a sustainable strategy for managing such waste and recovering nutrients which would otherwise be lost to the environment, representing a potential of source pollution;
- (iii) feed limitation can be effectively used for reducing the environmental impact of the insect-mediated waste bioconversion, promoting waste volume reduction while ensuring better nutrients sequestration and recovery;
- (iv) GHG emissions from BSFL grown on conventional feed can be traced back solely to biogenic CO₂, which is produced naturally in larval metabolism. GHG emissions are therefore strictly correlated with the insect growth and the final weight achieved. Additionally, as larval metabolism appears to be highly active during the first days of the insect life, correct estimation of the GHG emission from edible insects should consider the overall life cycle, from the eggs to the harvesting stage (or to the adult stage in case of individuals intended for reproduction).

Although these results provide valuable insight supporting the use of edible insects in the circular bioeconomy, contributing to a better understanding of the insect-mediated waste conversion process and defining new strategy for ensuring high quality of intermediate and final products intended for food and/or feed application, they should not be considered conclusive.

Further research efforts are required to gain a better understanding of the basic phenomena behind the obtained results. For example, although the combination HHP + MAP have shown to be beneficial within a long storage period, the immediate effect of HHP on the lipid oxidation of insect-based products is still unclear. Different intrinsic and extrinsic aspects such as insect species, rearing process, applied pre-treatment, and physicochemical properties of the initial material should indeed be considered and the effects of different treatment conditions deserve more attention.

Similarly, although BSFL have shown ability to grow on ASW, pure waste has displayed limitations, which may be associated with the extremely high water content or to the anaerobic pre-process happening during long-term storage (which is common in large-scale aquaculture facilities). As different strategies such as waste pre-treatment and waste co-bioconversion have shown high applicability to other types of waste, future studies should focus on the application of such technologies for ASW management. Moreover, the concept of reducing the environmental impact and improving the nutrients recovery from waste by applying feed limitation should be further explored

and validated, although not allowed according to the current legislation. Alternative uses, such as the extraction of functional compounds including antimicrobial peptides and/or antioxidants, do not seem to be strictly connected with the amount of generated insect biomass and should be further explored. A large range of applicability should finally be attributed to the two developed methods; fluorescence and gas detection chamber. Fluorescence spectroscopy might be further explored as a non-destructive and rapid method for online monitoring of quality changes in edible insects products, while the gas emission chamber could be applied on a larger scale to detect the GHG emissions from a wide range of insect species, as well as from the same species reared on different substrates. Generated knowledge should be further explored within the industrial sector to enhance the efficiency of various processes, reduce waste and identify the optimal combination of technologies and operating conditions. More advanced technologies, such as sensors, physical and mathematical models, machine learning and AI, are needed before applying these new technologies on a large scale. Combination of knowledge coming from diverse and seemingly distant fields will be necessary to effectively implement a true transition toward the circular bioeconomy.

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A1. Other applications of insects in the agrifood sector

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Other applications of insects in the agrifood sector

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1. Introduction

Humans have traditionally consumed insects as a supplemental protein source in various regions across the globe. In recent years, interest in entomophagy has rapidly increased as a potential sustainable resource. Edible insects are being considered as high value alternative protein sources for future, exhibiting high quality nutrient compositions along with potentially health-promoting macronutrients and micronutrients. Apart from proteins, insects can be a potential source of high quality fats, novel bioactive including bioactive peptides, chitin, phenols and vitamins. The demand for edible insects is increasing; therefore, a growing number of companies are entering the market to produce, process and market insect-based products. One of the important aspects of the use of edible insects is related to the environmental and ecological consideration. Insects can be reared on the food waste or low-value substrates, which provides an attractive key for bringing the losses back to the food chain (Ojha et al., 2020). Overall, insect farming requires less land, water, energy and feed than traditional livestock farming, and generates less greenhouse gas emissions.

Insect rearing involves a variety of methods and techniques, depending on the species, intended use and the scale of rearing. In general, insect farming involves creating a suitable environment for the insects to breed and grow, providing them with appropriate feed and water and controlling temperature and humidity to optimize their growth and development. Further, the processing of insects is paramount to deliver safe and high quality raw materials, ingredients and products for large-scale food and feed applications. Depending upon the nature of the initial material and the desired end product, the processing pathways vary and may include several unit operations currently used in food and feed processing. Insect processing pathways can involve harvesting, preprocessing, decontamination, further processing, packaging and storage. Several traditional and industrial decontamination methods have been proposed for edible insects, which include smoking, drying, blanching/boiling, marination, cooking, steaming, toasting and their combinations. Further processing steps are employed to produce insect meal, insect flour or extracted insect fractions. Each operation will have a different impact on the chemical and microbiological properties of the final product.

According to several global market insights, the global edible insects market is expected to rise in next decade, owing to increasing protein demand, climate policies, changing dietary needs and ongoing innovation in insect-based products (Mancini et al., 2022). The increasing list of approvals for different species of edible insects for human consumption in the EU is an indication of increasing popularity of insect-based products in Western world. In addition to use of edible insects as human food, they have potential applications in various other fields including animal feed, organic waste reduction/treatment and organic fertilizer.

This chapter outlines the nonfood applications of insects in the agrifood sector including as feed for aquaculture, poultry, livestock and pet animals. Application of insects for agrifood waste management along with challenges has also been reflected. Further, the chapter outlines the usage of insects in the agrifood chain including pest control, fertilizing, biofilms, edible coating, food additives and bioremediation. Finally, regulatory aspects and legislative framework connected with the use of insects and insect byproducts in the agrifood sectors have been discussed extensively.

2. Insects as feed

Currently, the livestock sector is the main responsible factor for the use of land, with the feed production being the primary source of environmental pollution (Kepinska-Pacelik & Biel, 2022). Insects may represent an interesting and sustainable alternative to traditional animal feed such as soybean meal and fishmeal. Recent studies have shown that several insects may meet the nutritional requirements of different animal species, being already part of their natural diet and being well accepted by them (Sanchez-Muros et al., 2014). However, the magnitude of the Class Insecta (Linnaeus, 1758) and the variability of the animals' requirements do not allow for a generic discussion, requiring a more detailed, species-specific analysis.

Among the insect species, the most promising for animal feed are the black soldier fly (*Hermetia illucens*), the yellow mealworm (*Tenebrio molitor*) and the common house fly (*Musca domestica*) (Sogari et al., 2019). The percentage of their inclusion in commercial animal feed depends on insect species and animals' requirements and should always consider the growth and production performances as well as the health status of the farmed animals. In view of this, it is quite improbable that insects will be able to replace the traditional feed completely, although they can still play an important role in animal nutrition. Additionally, whether insects are intended to feed food-producing animals, nutritional and sensorial aspects of the derived products should be considered.

2.1 Insects as feed in aquaculture

Aquaculture represents one of the most important sectors in the economy of several countries around the world (FAO, 2022). Commercial feed traditionally applied in aquaculture is based on fishmeal whose production, depending on the limited wild fish reservoirs, is environmentally and economically unsustainable (Alfiko et al., 2022). Novel protein sources such as soybean meal or other plant-based materials have been investigated; however, due to their poor nutritional quality and high content of antinutrients, they do not seem to be suitable solutions for fishmeal replacement (Daniel, 2018). On the other hand, insects display a nutritional composition closer to fishmeal; therefore, they might be more suitable candidates for being used in aquaculture (Alfiko et al., 2022). Several insect species including *H. illucens*, *T. molitor*, *Zophobas morio*, *Bombyx mori*, *M. domestica*, *Grylodes sigillatus*, *Gryllus assimilis*, *Blatta lateralis*, *Nauphoeta cinerea*, *Gromphadorhina portentosa*, *Lucilla sericata*, *Cirina butyrospermi*, *Imbrasia belina*, besides chironomidae and locusts, have been evaluated in fish feeding trials (Liland et al., 2021). Among these, black soldier fly (BSF) larvae and yellow mealworm represent the two most probable species to becoming common in aquaculture (Aragao et al., 2022). When insect meal is used as fishmeal replacement, growth performances, as well as haematological parameters and healthy status of fish could be affected, with results strictly depending on insect species, aquatic species and percentage of inclusion. Generally, levels of insect meal higher than 30% led to reduction of fish final weight, while no significant effects were observed in terms of feed conversion ratio and positive consequences were detected on fish health (Hua, 2021; Liland et al., 2021; Maulu et al., 2022). Possible explanations might be found on the particular nutritional composition of the insect meal. Insects are rich in chitin that, although not usually digested and absorbed by fish (Sanchez-Muros et al., 2014), exercises a positive healthy effect showing antiinflammatory and immune-stimulant activities (Maulu et al., 2022). Moreover, most of the insect species are characterized by low levels of sulphur amino acids which are known to be essential for several fish species (Alfiko et al., 2022; Luthada-Raswiswi et al., 2021), extremely low ash content (especially calcium) and a calcium/phosphorous ratio lower than 1.1 (optimal for most of the fish species) (Alfiko et al., 2022). Moreover, the fatty acid profile of insects deserves also further attention. Although it can be easily manipulated with the diet (Oonincx et al., 2020; Rossi et al., 2022; St-Hilaire et al., 2007), insects are usually deficient in polyunsaturated fatty acids such as EPA and DHA (van Huis & Gasco, 2023).

Results in terms of growth efficiency are extremely variable, depending on aquatic species, as well as on the insect species. For example, carnivorous fish species, such as seabream, salmon, rainbow trout and common catfish usually display higher acceptance for dietary insects when compared to omnivorous fishes as eel, carp, Nile tilapia, Siberian sturgeon, and perch, and crustaceans (Alfiko et al., 2022; Liland et al., 2021). Differences in performances have been also recorded when the same fish was fed with different insect species. For example, mealworm are usually better accepted by fish than BSF (Hua, 2021), although total replacement of fishmeal with BSF larvae meal was possible in Atlantic salmon (Belghit et al., 2018). Similarly, substitution of 100% of fishmeal with silkworm pupa meal without affecting growth and production performances was possible in common carp and seabass, while the same insect species at 5%–10% of inclusion yielded very low performances in Jian carp and tilapia (Alfiko et al., 2022).

2.2 Insects as feed for poultry

Insects represent a natural feed for poultry (Elahi et al., 2022). Free-range animals are usually found picking worms from the ground that may not just represent a nutritive source, but also stimulate their immune system by exercising an immunomodulatory effect (Malematja et al., 2023). Insects are rich in chitin, antimicrobial peptides, functional proteins, lauric acid and other bioactive compounds, which may enhance the intestinal microbiota, reduce serum cholesterol, promote the growth and stimulate the animal immune system, granting more resistance to pathogens and improving their health status (Dorper et al., 2021; Malematja et al., 2023).

Several insects species, such as *H. illucens*, *T. molitor*, *M. domestica*, *Macrotermes bellicosus*, *Rhynchophorus ferrugineus*, *B. mori* and several crickets, locusts and grasshoppers, have already been tested as feed source for broilers, hens, quails, partridges, turkeys and guinea fowls (Adli, 2023; Dorper et al., 2021; Moula & Dettleux, 2019). Generally, although insects are well tolerated by birds (Elahi et al., 2022), inclusion levels higher than 10% should be avoided, since at higher value, feed conversion ratio and daily weight gain decrease, while feed intake significantly increase (Moula & Dettleux, 2019). Possible explanation for this might be the reduced digestibility of the feed containing high level of insect meal, as well as the unbalanced amino acid profile of most of commercially available insect species (Elahi et al., 2022; Moula & Dettleux, 2019). Although poultry have ability to digest chitin (Lee et al., 2022), high amount of chitin may significantly reduce the digestibility of the overall feed leading to reduced growth and production performances. Moreover, diet based on insect meal might be poor in lysine and methionine (two crucial amino acids for poultry), which should be artificially supplemented, depending on the animal requirements (Elahi et al., 2022; Veldkamp & Bosch, 2015). High inclusion of insect meal in poultry feed has been recommended in multiple studies since insects may stimulate poultry growth, enhance production performances, ensure better health status and improve the products quality (Edea et al., 2022; Elahi et al., 2022; Malematja et al., 2023; Shah & Çetingul, 2022). However, it is important to note that in some cases, high level of insect meal in poultry diet might also be responsible for negative effect on the morphology of the animal gut. For example, reduction of the intestinal villi and consequently reduction in intestinal absorptive area have been observed in laying hens and broilers fed with more than 10% (of the overall diet) of mealworm and BSF meal (Biasato et al., 2018; Moniello et al., 2019).

2.3 Insects as feed for pigs

Pig farming is mainly based on cereals and soybean, with the last making approximately 20% (dry matter bases) of the overall feed and representing the main protein source for pig (Parrini et al., 2023). Although the use of alternative resources such as insects is not new in pig diets, only few studies have been published so far. Despite some contradictory results, depending on the insect species and life stage as well as on age and physiological status of the fed animal (Veldkamp & Vernooij, 2021), general trend shows that insects in whole or defatted form can be included in pig diets as full or partial replacement for soybean meal (Veldkamp & Vernooij, 2021). No adverse effects in terms of weight gain, feed conversion ratio and nutrient digestibility have been observed in growing pigs fed with diets containing up to 20% *H. illucens*, *M. domestica* or *T. molitor* meals. On the other hand, BSF inclusion levels of 5% in diets of weaned piglets resulted in poor nutrient digestibility and high oxidative stress, although growing performances were not directly affected (Szczepek et al., 2023). However, 10% of mealworm meal in weaning piglets diet did not display any adverse effect (Hong et al., 2020). Interestingly, inclusion of 4% dried BSF powder resulted in higher final weight and lower feed conversion ratio than traditional feed in finishing pig, which were also characterized by better meat quality with higher juiciness, higher polyunsaturated fatty acids and bigger loin-eye area (DiGiacomo & Leury, 2019).

2.4 Insects as feed for ruminants

Although the use of insects in ruminants (goat, cattle and sheep) diet has been far less investigated than in monogastric animals, recent studies have shown that they might be conveniently used in ruminants feed (Castillo & Hern, 2023). Insect meal derived from *Gryllus bimaculatus* and BSF larvae have shown interesting results as milk replacer (30% of the overall formulation) for preweaning goat (Astuti & Wiryawan, 2022; Renna et al., 2022). In postweaning ruminants, diets containing 25% of *Acheta domesticus*, *G. bimaculatus*, *G. sigillatus*, *Alphitobius diaperinus*, *M. domestica*, *T. molitor*, *Brachytripes portentosus*, *B. mori* or *H. illucens* as replacement of soybean meal did not show any adverse effect on nutrient digestibility or rumen fermentation while reducing the methane production (Ahmed et al., 2021; Renna, Coppa, et al., 2022; Renna, Rastello, et al., 2022). When insect meal were included in ruminant diet, an increase in ammonia emission was observed (Ahmed et al., 2021); however, it was not confirmed in other studies (Renna, Coppa, et al., 2022). Such differences between studies could be explained by the different level of insect meal inclusion in the feed. Insects are rich in fat which may have detrimental effects on rumen fermentation, reducing the digestibility of feed (Ahmed & Nishida,

2023). Furthermore, the chitin contained in the insects meal, although able to modulate the digestion process, enhances the bacterial diversity and improves the rumen fermentation (Shah et al., 2022), in high concentration may reduce the overall digestibility of the feed, leading to lower protein degradation and lowering ammonia and volatile fatty acids' production. It might result in low productive performance whether the animal has not an efficient gut digestion mechanism. Therefore, insect meal inclusion higher than 20% of the overall ration is usually discouraged (Ahmed & Nishida, 2023).

2.5 Insects as pet food

Insects are part of the natural diets for many canids and cat species (Kepinska-Pacelik & Biel, 2022). For reared pets, use of insects as feed mainly depends on the owner willingness and culture, whom, considering the pet as family member, tends to buy the best feed material for the own pet (Bosch & Swanson, 2021; Valdes et al., 2022). Although there is great interest in using insects as pet food, limited data is available in literature so far. These are usually recorded on very small number of subjects (2 or 3) and/or over few days' trial, masking the real effect of insects inclusion (Bosch & Swanson, 2021). *T. molitor*, *H. illucens* and *A. domesticus* are the most studied species as pet food (Valdes et al., 2022). Most of the trials performed on dogs or cats showed that dogs well accepted dry food containing up to 10% of BSF larvae meal (Bosch & Swanson, 2021) or 24% of *G. sigillatus* meal (Kilburn et al., 2020), while cats did not like feed containing more than 5% of BSF larvae meal (Valdes et al., 2022). Digestibility of insects containing diets ranged from 75% (with BSF larvae meal) to 85% (with mealworm meal) for dogs and between 75% (for BSF) to 80% (for yellow mealworm) for cats (Beynen, 2018; Lisenko et al., 2023). Similar values were recorded for commercial pet food (Bosch & Swanson, 2021). Stool consistency, a parameter often associated to intestinal health, was not impacted by insect inclusion in diet (Bosch & Swanson, 2021; Lisenko et al., 2023; Seo et al., 2021), suggesting that the indigestible fraction of the insects (e.g., chitin) was not fermented by the animal microbiota, being found in the faeces (Bosch et al., 2016). Some examples of insects-based feed for dogs and cats are presented in Fig. 13.1.



FIGURE 13.1 Examples of dog and cat feed containing insects. Source: Valdes et al. (2022).

3. Insects for agrifood waste management

3.1 Waste in the agrifood chain

Food waste can be defined as leftover food that is generated in the agrifood chains (Edjabou et al., 2016). The global amount of the edible food waste that is generated every year is estimated to be approximately 1.3 tons, which equals to

almost one-third of total food production (Mishra et al., 2022; Roy et al., 2023). Food waste has been categorized as avoidable, partly or optionally avoidable and unavoidable (Kranert et al., 2012). At an industrial level the avoidable food waste corresponds to contaminated or damaged food, while at household level the avoidable food waste includes the food that is wasted due to poor storage, overpurchasing and inappropriate preparation techniques (Bagherzadeh et al., 2014). The partially avoidable food waste is related to consumer habits, as for instance apple peels, while the unavoidable food waste refers to nonedible parts of a food product, e.g., banana peels (Shikha Ojha et al., 2020).

It is considered that in the EU the majority of the food waste is generated at household or consumer levels (Stenmarck et al., 2016). However, food is wasted throughout all stages of the food chain (Edjabou et al., 2016). In the production stage, food can be wasted due to pests, diseases, damage in harvested products and extreme weather. At the postharvest stage, food can be wasted due to poor storage and handling, while at a processing stage, food can be wasted due to process failure, rejection of final products, contamination or defect raw materials. Finally, at the distribution stage, food is wasted due to overstocking, spoilage, outdated products and changes in demand (Edjabou et al., 2016; Food & Nations, 2017; Ojha et al., 2020).

3.2 Insect-based bioconversion of waste

Edible insects have been underlined for their potential to close the cycle in circular economy due to their ability for waste bioconversion (Ojha et al., 2020). In particular, food wastes and side-streams can be introduced to edible insect diets as feeding substrates or dietary components (Varelas, 2019), thus reducing the amount of waste as part of an insect bio-refinery, where insects are then converted into products in the agrifood and feed, cosmetic, pharmaceutical and biodiesel chain (Leni et al., 2021). In some cases food waste can be utilized as insect feed individually, as for instance brewers' spent grain and restaurant kitchen waste were used directly as feed for BSF larvae in Kenya (Shumo et al., 2019). Furthermore, byproducts from the production of different cereals and legumes in Greece have been successfully utilized individually as feed for larvae of yellow and lesser mealworms (Rumbos et al., 2021), while agro-byproducts in Kenya have been shown to have potential as a rearing substrate for crickets (Orinda et al., 2017).

However, food waste can also be used as ingredients of insects' diets, where the composition of the final diet is considered to address the nutritional requirements of the insects, as suggested for different species of crickets (Kuo & Fisher, 2022). These requirements refer mainly to the content of the feed in proteins, essential amino acids, carbohydrates, minerals and polyunsaturated fatty acids, while different insect species have different requirements in nutrients (Kuo & Fisher, 2022; Leni et al., 2021). For instance, byproducts that are generated in Greece from the production chain of peas, vetch, oat, barley, sunflower and cotton could be utilized for the rearing of *T. molitor* larvae both as diet ingredients and individually to reduce feed costs (Rumbos et al., 2022). Additionally, plant based byproducts have been successfully used as diet ingredients for the mass rearing of cricket species, such as *A. domesticus* and *G. bimaculatus* (Sorjonen et al., 2019). In some cases, even though insect diets that were based on organic byproducts have been underlined for the potential for future utilization, they did not lead to an acceptable yield of the insects in question (*Z. morio*, *T. molitor*, *A. domesticus*, *Locusta migratoria*) (Harsányi et al., 2020; Jucker et al., 2022; Van Peer et al., 2021). Finally, the inclusion of food waste in the diet of edible insects can have a potential positive effect on its composition, as in the case of *T. molitor* that showed an increase in the content of proteins and vitamins A and C after including orange peels in its diet (Kotsou et al., 2023).

3.3 The case of BSF

Among the insect species that are considered for agrifood waste bioconversion, there is a major interest in the *H. illucens*. This particular interest is motivated mainly by the ability of BSF larvae to grow on a wide variety of wastes, such as food leftovers, manure, human faeces and catering and municipal waste (Gold et al., 2020; Surendra et al., 2020). However, the kind of waste, e.g. vegetable or fruit waste, municipal waste or milling and brewery waste, can affect the weight and survival rate of the pupae, the development time, as well as the percentage of waste reduction and the rate of bioconversion (Gold et al., 2018). For example leftovers of different foods (exotic fruits, pineapples, kiwi, apples, melons, tomatoes, peaches, pomace, legume and corn) were used for the preparation of mixtures for diets for BSF larvae, with the insects grown in all designed diets showing development, but with different individual weight and number of pupae depending on the diet (Barbi et al., 2020). The digestion process of BSF larvae has been described in literature. After the larvae ingest the feed, it goes through the foregut (oesophagus and proventriculus) to the midgut (anterior, mid and posterior). The midgut is the most important part of the larvae digesting system and is the part where the feed is decomposed due to the environment of the gut (oxygen and pH), enzymes and microorganisms. The nutrients can be stored in the fat tissues of the larvae after decomposition. They can also be passed on to the haemolymph, which is the blood equivalent of the larvae and transfers

the nutrients to the whole body. After the midgut, the diet goes to the hindgut and finally the metabolic wastes and the nondigestible molecules are excreted (Gold et al., 2018).

3.4 Animal manure

Edible insects have also been suggested as a possible management method of animal manure, which is a major waste of livestock production and its already existing management methods are considered insufficient or not fully sustainable (Cammack et al., 2021). Many species are good candidates for upcycling animal manure since they have been reported to feed naturally on manure (Miller, 1954; Mohr, 1943; Nichols et al., 2017). However, the ones considered particularly promising are the lesser mealworm (*A. diaperinus*), the housefly (*M. domestica*) and the black soldier fly (*H. illucens*) (Cammack et al., 2021). Houseflies are especially valuable because they can digest large volumes of manure. In specific, they can digest over 100 kg of swine manure in 1 week (Zhang et al., 2012) and over 400 kg of swine manure in 2 weeks (Čičková et al., 2012). However, similarly to lesser mealworms, they are considered pests and pathogen vectors (Keiding & Organization, 1986).

The use of BSF larvae is very promising for animal manure management as part of a circular bioeconomy (Liu et al., 2022). They can digest high amounts of manure of cows, pigs and poultry, with the manure from poultry being the most efficient for the larvae growth (Diener et al., 2009; Miranda et al., 2019; Zhou et al., 2013). They have also been reported to grow on cow manure combined with fish offal (St-Hilaire et al., 2007) and they offer the advantage that they are not considered pests (Alyokhin et al., 2019; Lalander et al., 2019). BSF larvae can even be used to degrade the byproduct of the rearing of locusts and two species of crickets (frass), with the flies showing a higher development and waste reduction rate when reared on the frass from crickets (Jucker et al., 2020). BSF have also shown potential for utilizing aquaculture sludge, which contains faeces, uneaten feed and biofilm from biofilters. In the case of sludge from juvenile Atlantic salmon, BSF larvae are shown to grow on a sludge including feed and accumulate heavy metals (Liland et al., 2023).

3.5 Pretreatments to improve bioconversion of waste

Agrifood wastes are commonly lignocellulosic materials that they are difficult to utilize for insect bioconversion due to their structure, e.g. lignin content, insolubility and resistance to depolymerization (Agbor et al., 2011; Peguero et al., 2022). However, the bioconversion of agrifood wastes can be improved by physical, chemical and biological pretreatments (Peguero et al., 2022).

Physical pretreatments refer to mechanical, thermal and electromagnetic processes that alter the structure of a biomass (Atelge et al., 2020). Mechanical processes are mainly chopping and milling that aim to reduce particle size (Oyedemi et al., 2020), which has been correlated to improve biogas and methane production (Victorin et al., 2020). Thermal pretreatments refer to processes of biomass at a range of elevated temperatures (>100°C in some cases) for a time of 15–120 min (Atelge et al., 2020). They are used in order to improve the solubility of organic matter, enhance biodegradability and facilitate the production of biogas and composting (Appels et al., 2010; Cesaro & Belgiorno, 2014).

Chemical pretreatments refer to the processing of a biomass with oxidizing agents, acidic or alkaline solutions and ozone (Behera et al., 2014). The aim of chemical pretreatments is to disrupt the structure of lignin and hemicelluloses in order to enhance their accessibility. The efficiency of these pretreatments depends on the structure and composition of the biomass, chemical medium and processing conditions including time and temperature (Peguero et al., 2022).

Biological pretreatments refer to the mixing of biomass with bacteria, yeasts, fungi or a combination of microorganisms that can degrade lignin and cellulose due to microbial enzymes (Atelge et al., 2020; Galbe & Zacchi, 2012). The efficiency of biological pretreatments depends on the substrate, type of microorganism, processing conditions, e.g. temperature and time and carbon and nitrogen sources (Sharma et al., 2019). Even though the aim of biological pretreatments of agrifood wastes is similar to the ones of physical and chemical pretreatments, biological pretreatments show major environmental advantages, such as lower energy requirements and reduction of use of hazardous chemicals. However, the biological pretreatments for decomposition of biomass is slower, while being harder to control the process (Peguero et al., 2022).

Waste pretreatments have been considered promising for improving the ratio of waste conversion to biomass by insects (Peguero et al., 2022). For instance, in the case bioconversion of banana peels by BSF larvae, a microbial pretreatment, a nonprotein nitrogen pretreatment and nonprotein nitrogen and microbial pretreatment could improve the conversion ratio, in respect to nonpretreatment, as opposed to plain thermal treatment and combined thermal and microbial pretreatment (Isibika et al., 2019). However, the application of processing methods for an enhanced waste bioconversion by insects is still limited.

3.6 Challenges and future perspectives

The bioconversion of agrifood waste with insects is receiving a growing interest among researchers and stakeholders. However, there are a number of challenges that need to be addressed, which are related to contaminants found in these waste materials, such as heavy metals, mycotoxins and pesticides (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022; Van der Fels-Klerx et al., 2018). In this regard, BSF are particularly interesting, because they can ingest many food contaminants without having their growth and survival affected (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022). They have been shown to accumulate heavy metals, e.g. cadmium, arsenic and lead, while excreting mycotoxins and pesticides and reducing their initial concentration in the frass (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022; Purschke et al., 2017). However, the accumulation of these contaminants depends on the insect species and life stage (Van der Fels-Klerx et al., 2018).

Nevertheless, agrifood waste bioconversion with insects has been underlined for its environmental sustainability. Life cycle analyses have shown the lower greenhouse gas emissions and global warming potential of generated insect biomass, as for example BSF, when grown on side-streams (Smetana et al., 2019), with the energy consumption being one of the major issues still to be addressed (Salomone et al., 2017).

4. Further use of insects in the agrifood chain

4.1 Use of insects in organic farming for pest control

Organic production of crops is a major segment in agriculture (Zehnder et al., 2007). Its worldwide application is an answer to the environmental and health concerns regarding the synthetic pesticides (Tsvetkov et al., 2018). Exposure to pesticides even at small amounts has been linked to toxic effects to humans, such as skin irritations, allergic reactions and nausea, while some can be lethal (Damalas & Koutroubas, 2016). Additionally, pesticides have been reported to cause environmental hazards, such as surface and ground water contamination via runoff from plants, soil contamination, reduction of soil fertility due to a declination of beneficial soil microorganisms, while herbicides could also be found in nontarget organisms, as for instance nontarget plants, fish and birds (Aktar et al., 2009).

Organic farms are reported to rely on biological control (Zehnder et al., 2007), which can replace synthetic pesticides with integrated parasitoids and predators to control insect pest populations at acceptable levels (Cloyd, 2020) and at the same time address the decline in global insect populations (Galloway et al., 2021). For example, a comparison between conventional and organic farming in the case of California tomatoes showed that the organic farm was richer in predatory arthropods, with the withdrawal of synthetic pesticides not affecting the insect-induced damage to the plants (Letourneau & Goldstein, 2001). Similar results were reported for rice farming in the city Patiala in India, where organic rice farms were found to contain higher amounts of beneficial predators, such as spiders, dragonflies and damselflies (Sharma et al., 2020).

Natural enemies, such as parasitoids and predators are a good strategy for farmers, who can conserve them for biological control in a simple and cost-effective way, with a low effort being required when already available indigenous natural enemy species are utilized. Some crops that have already been reported to utilize parasitoids are sugarcane, rice, cotton, tobacco, coconut, apple, citrus and tomato (Ballal & Verghese, 2015). It has also been reported that particular plants can be used to attract natural enemies by providing pollen and nectar, e.g. Michigan native plants (Fiedler & Landis, 2007). Wildflowers can be also used to attract predators, such as spiders, hoverflies and lady beetles to control the number of earworm eggs, as in the case of adjacent blueberry fields (Blaauw & Isaacs, 2015).

However, the application of biological control in organic farming via the use of parasitoids and predators has significant challenges. The appropriate combination of species has to be identified and applied to obtain the desired farming results without a major impact on nontarget species and at the same time ensuring environmental heterogeneity (Ballal & Verghese, 2015; Galloway et al., 2021).

4.2 Insect frass as organic fertilizer

The production of edible insects generates a large amount of organic waste that consists of faeces (also referred to as frass), exuviae and uneaten feed (Ojha et al., 2020). The amount of generated frass is particularly large, reaching thousands of tons per year (I. P. o. I. f. Food & Feed, 2019) and being 40 times larger than the production of animal biomass (Jorge Poveda, 2021). For example in the case of BSF larvae, 1000 kg of feed substrate can yield 250 kg of frass (25%) (Basri et al., 2022).

Insect frass has been underlined for its potential for utilization as fertilizer, due to its valuable content in minerals and microorganisms that can facilitate growth and nutrient intake of plants and due to its generation of tolerance to abiotic stress (I. P. o. I. f. Food & Feed, 2019; Jorge Poveda, 2021). In particular, the evaluation of the mineral composition of frass from several edible insects including *H. illucens*, *T. molitor*, *B. mori* and *G. bimaculatus* has shown their rich composition in valuable minerals. The amounts of these minerals such as nitrogen, phosphorus, potassium, calcium, zinc, magnesium, iron, manganese and copper differ depending on the insect species (Beesigamukama, Subramanian, et al., 2022). Additionally, bacteria that can improve plant health have also been identified in insect frass, with varying fertilizing potential, depending on the insect diet, as suggested for mealworms and BSF (Fischer & Romano, 2021; Poveda et al., 2019; Romano et al., 2023).

The use of frass as fertilizer has been tested and proven promising in some cases. For example, frass from BSF can increase maize grain yield compared to mineral fertilizer (NPK) or brewers' spent grain, while having economic benefits for farmers (Beesigamukama, Mochoge, et al., 2022; Tanga et al., 2022). Additionally, BSF frass compost from insects that grew on food waste and human faeces leads to comparable yields of Swiss chard to inorganic fertilizer (Chirere et al., 2021). Despite the promising potential of frass as fertilizer, there is a need for its better matrix stabilization via processing or combination with other fertilizing materials (Lopes et al., 2022).

4.3 Insects for edible films and coatings

Edible films and coatings are synthesized by the combination of plasticizers and food grade biopolymers, e.g. proteins, polysaccharides lipids. They are thin layers that are applied to the surface of a food product to protect it from physical, chemical and biological degradation and extend its shelf life (Banks et al., 1993; Dhall, 2013; Han, 2014; Park, 1999).

Edible coatings can function as a barrier to oxygen, moisture and solute moving (Baldwin et al., 2011; Dhall, 2013). They find many applications in fruit and vegetables, because these foods respire after harvesting. Coatings can increase the foods' resistance to gas diffusion and alter their internal atmosphere composition, shifting them to partial anaerobic respiration. Consequently, ethylene production, which accelerates ripening, is disrupted and fruits and vegetables can remain fresh, nutritious and with an increased shelf-life (Dhall, 2013; Guilbert et al., 1996; Park et al., 1994). However, edible coatings are applied to meat and fish, as well, in order to reduce moisture loss that would negatively affect sensorial characteristics of the products, prevent lipid oxidation and microbial growth, prevent volatile flavour loss and improve quality during cooking by preventing oil uptake during cooking methods like frying (Gennadios et al., 1997).

Edible insects have been suggested as the source of components for production of edible coatings and bio-based packaging materials. Chitosan films have been produced from *A. domesticus* and *G. sigillatus* that can improve the water resistance and light barrier properties (Malm et al., 2021). A protein blend from grasshoppers and soy when combined with xylose and cinnamaldehyde could generate an edible film that can resist *Escherichia coli* and *Staphylococcus aureus* and reduce water vapour permeability (Zhang, Zhou, et al., 2022), while proteins from migratory locusts have been underlined for their potential for implementation in edible films (Zhang, Fang, et al., 2022). However, the knowledge on edible films and coatings from insects is still limited.

4.4 Insects for food additives

One of the major advantages for utilising edible insects in the food sector is their high nutritional value (Rumpold & Schlüter, 2013) and they can be consumed in various forms, such as fresh, frozen, dried, fermented, powder, but also as food ingredient (Ojha et al., 2021). Consumers without tradition in entomophagy tend to disapprove edible insects due to aversion (Mishyna et al., 2020). Therefore, one emerging application of insects as food is their use of nonvisible food ingredients, as for example house crickets have been used as ingredients in sourdough (Rossi et al., 2021) or cereal bars (Ribeiro et al., 2019).

In this aspect, edible insects can be considered as source of food additives, especially since they are reported to contain antioxidants, such as phenolic compounds (Nino, Reddivari, Osorio, et al., 2021; Nino, Reddivari, Ferruzzi, et al., 2021), and nutrients, such as vitamins (Oonincx & Finke, 2021). One case of application of food additives from insects is carmine, which is a red dye produced from carminic acid that is extracted from cochineals that produce it as a defence mechanism from predators (Eisner et al., 1980, 1994). Carmine finds application in various sectors, including the food sector (Dapson, 2007) and has been identified in foods, such as ice cream, soft drinks, strawberry flavoured milk and candies (Alghamdi et al., 2009; Yilmaz et al., 2014).

Another case of use of insects as food additives is the generation of oil in water emulsions from ingredients from house crickets, where cricket protein and chitin fractions have been investigated for their emulsifying capacity (Hirsch et al.,

2019). Additionally, cricket-based proteins can be used to produce stable emulsion when combined with emerging food processing technologies, e.g. ultrasound and high pressure (Han & Heinonen, 2022). However, the number of studies focussing on the potential of producing food additives from insects is scarce.

4.5 Insect bioremediation

One further proposed application of insects in the agrifood chain is the use of insects for the purpose of remediation. Collembolans, ants, beetles and termites are considered valuable candidates for degraded soil treatment due to their nutrient cycling and soil aerating abilities, contributing to an insect-based remediation process called ‘entomoremediation’ (Ewuim, 2013). The BSF larvae is a particularly interesting species for this application because it is shown to be able to consume contaminants and accumulate heavy metals (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022). Additionally, BSF larvae have been successfully used to reduce the amount of polluted biomass from corn leaves, which contained considerable amounts of cadmium, by almost 50% (Bulak et al., 2018). Another example of insect-based bioremediation is the expression of fungal laccase in *Drosophila melanogaster* for the treatment of bisphenol A, which is a waste produced by the plastic industry, with the fly degrading more than 90% of bisphenol A (Clark et al., 2021).

Finally, insects have shown potential for degrading plastic, like polystyrene and polyethylene, due to their gut microbiota (Khan et al., 2021). This application has been mostly explored for species like yellow mealworm (Brandon et al., 2018; Yang et al., 2018), superworm (Koh et al., 2020; Peng et al., 2020) and wax moth larvae (Lou et al., 2020). This application of insects is proposed as an answer to the environmental impact of plastic waste, since it has been calculated that up to today more than 8000 million metric tons of plastic have been produced (Geyer, Jambeck, & Law, 2017).

4.6 Insects as a source of energy

The increase of human population in the future is expected to cause an increase in the demand for resources, such as food and energy (Surendra et al., 2015). Insects have been the focus of interest in this aspect, due to their ability to generate fat-based biomass that can be utilized for biodiesel production (Nguyen et al., 2019; Su et al., 2019) and can be used as basis for an ether extract that can reach up to 77% (Manzano-Agugliaro et al., 2012).

As it was previously discussed in this chapter, edible insects have waste bioconversion potential (Ojha et al., 2020). In this perspective, insects can be produced by feeding on agrifood waste substrates and then utilized as lipid sources for production of biodiesel (Surendra et al., 2016; Wang et al., 2017; Yang et al., 2014; Zheng et al., 2012). For instance, mealworm and BSF larvae have been suggested for bioconversion of crop residues to proteins and biodiesel (Wang et al., 2017). BSF have been grown on restaurant or cafeteria waste (Surendra et al., 2016; Zheng et al., 2012), while houseflies can be reared in pig manure (Yang et al., 2014) waste for the purpose of producing biodiesel.

5. Regulatory aspects

Although insects are interesting and have been considered for different purposes in the agrifood system, not all the aforementioned applications are allowed. Legislation limits, including the topics related to animal welfare, have greatly reduced the application of these organisms, whose potentiality has not yet been fully understood by the legislator.

5.1 Animal welfare in insects rearing

One of the main aspects pushing the consumers away from consuming animal proteins is the ethical concern about animal rights and welfare (Ghaffari et al., 2022). Such concern raises from the idea that animals have feelings and therefore can experience stress and pain (van Huis, 2021). Although it is considered to be true for vertebrates such as Mammalia and Fishes (Harrison, 1991), it has been not completely understood in insects and other arthropods so far. Scientific evidence seems to suggest that insects possess receptors sensitive to potentially harmful stimuli and can react to traumas due to their innate behaviour, albeit they keep behave normally after being extremely injured (Eisemann et al., 1984). Recent studies have displayed that insects, like mammals, possess neural connection for processing noxious stimuli (Li et al., 2020), suggesting that they may experience pain. Such speculation was further supported by Gibbons et al. (2022), who evaluated the ability of several insect species to feel pain through eight different criteria. In that review, it was highlighted that, although the limited amount of studies did not allow exhaustive conclusions, there is no evidence that insects do not experience pain. Because of it, the precautionary principle (‘absence of evidence is not evidence of absence’) should be

applied, indicating that insects should be considered as sentient animals (van Huis, 2021). It follows that as stated by the International Platform of Insects as Food and Feed all insect producers should apply high standard of animal welfare through compliance with Brambell's five degrees of freedom (IPIFF, 2019). Adherence to these principles also allows to place the insects in the best condition for obtaining the best production possible, although rearing settings not always respect their 'natural behaviour'. A particular point of concern should then be the killing step. A first problem is related to the amount of killing. As the insect industry becomes bigger, a very large number of insects are killed (Rowe, 2020). Although the human empathy for invertebrates is very low, the question should be if killing thousands of insects makes more sense than killing only one vertebrate (van Huis, 2021). A second problem stands on the killing procedure. Although there is not specific rule for performing it, fast and effective methods are always preferred. Freezing, heating or quick shredding seems to be the suitable procedure, albeit inactivation by cooling before freezing or anesthetization with carbon dioxide or vacuum before blanching is considered more 'human' (Grabowski et al., 2020; van Huis, 2021). However, with some rare exceptions (such as the Finnish Food Safety Authority, the Thai National Bureau of Agricultural Commodity and Food Standard, the Dutch Animal Act, the German animal protection law and the Malaysian Animal welfare act 772/2015), no specific legislation is nowadays available about insect welfare (Delvendahl et al., 2022; Said & Bae, 2019). Moreover, the legislation standing for other reared animals, such as the EU Reg. 2004/884 and 2017/625, the Council Directive 98/58/EC and the Malaysian Animal Act 647/2006 does not apply to insects (IPIFF, 2022; Said & Bae, 2019; Zuk-Golaszewska et al., 2022), for whom, specie-specific guidelines are required (Delvendahl et al., 2022).

5.2 Insects for waste management

Ecological sustainability and insects' ability of growing on waste and byproducts is one of the aspects triggering the edible insect sector (Section 3). Despite such ability, legislative framework in several countries does not allow to rear insects on such material (Table 13.1). Especially when insects are expected to be used for food or feed production, they are considered as farmed animals and therefore their production should respect all the restrictions valid for every other animal (Bosch et al., 2019). For example, in Europe the regulations (EC) 767/2009 and (EC) 178/2002 state that animal feed should be safe for animal and environment, limiting the amount of material allowed for feeding insects to the feeding sources listed in Regulation (EU) 68/2013 and compliant to Regulation (EC) 1069/2009. Therefore, human or animal manure, sewage sludge, packaging material, food waste generated by restaurants, catering and households, byproducts from slaughterhouses which do not enter the food chain, animal carcasses and meat/fish no longer suitable for human consumption are not allowed as feed for insects. On the other hand, food waste generated from food not anymore suitable for human consumption (because expired or affected by packaging defects), residual streams containing animal byproducts (e.g., fishmeal) as well as vegetable waste (e.g., fruit peels, vegetable waste from industrial process, fermentation waste, molasses, sugar syrup), seeds and gardening/forest materials (as long as they are not treated with chemical or biological products in accordance with Reg. (EC) 767/2009, Annex III), can be used for feeding insects (Van Raamsdonk et al., 2023). Similarly, due to food and feed safety issues, use of feed contaminated materials (e.g., mycotoxins or heavy metals contaminated grains) is not allowed for rearing insects (Reg. (EC) 183/2002; Directive 2002/32/EC). On the other hand,

TABLE 13.1 Waste and byproducts allowed for rearing not food producing insects according with the legislation of different countries.

Country	Manure	Vegetal food waste	Animal food waste	References
Europe	X	✓	✓/X	Van Raamsdonk et al. (2023)
Africa	✓	✓	✓	Niassy et al. (2022)
North America	X	✓	X	Ominski et al. (2021) (Canada); Lahteenmaki-Uutela et al. (2021); Leib et al. (2016) (USA)
South America	✓	✓	✓	Mexico (2008) (Mexico); Allegretti et al. (2017) (Brazil)
Asia	✓	✓	✓	Bai et al. (2021) (China); Mukherjee et al. (2023) (India)
Australia	X	✓	X	https://www.sfmca.com.au/integrity-systems

whether insects are not intended for producing food or feed, different and less restrictive legislation (e.g., Commission Directive 82/475/EEC for pet food) applies, although it should still be kept on mind that highly contaminated materials like manure are always banned (Van Raamsdonk et al., 2023).

Less strict legislation is applied in Asian and African countries. In China for example animal manure might be used as feed under certain limitation, mainly concerned with its quality and safety (Bai et al., 2021). Although most of the farmers are not confident on using manure reared insects for feeding food producing animals, BSF larvae-mediated manure management is heavily promoted by the government, which also incentivizes farmers to use manure-fed insects as feeding source for livestock (Bai et al., 2021). About Africa, no clear list of admitted feed is present in most of the countries. It follows that all the materials might theoretically be used for feeding insects as long as their contamination with microorganisms, veterinarian drugs or other chemical substances which may pose a risk for animal health, is not higher than certain limits (Government of South Africa, 2009). However, when insects are not intended for human or animal consumption, no limitation on substrates are applied (Niassy et al., 2022).

Concerning the American continent, Canada government seems to be widely open on the possibility to use insects for food waste recycling, although not all the waste are currently admitted (Ominski et al., 2021). US legislation is quite wide, encompassing several regulations from several organisms, which are summarized in the Leftovers for Livestock Legal Guide (Leib et al., 2016). According with US legislation, in order to ensure safety for animals and humans, every reared animal species should be treated separately. Specifically, while food waste is allowed after being opportunely treated and declared microbiologically safe for swine as well as for pet nutrition, it is not allowed for ruminants. Insects are not explicitly included in the current US legislation, however, based on several manufacture practices, they can be reared on food waste as long as such material is safe and does not contain deleterious substances (Lahteenmaki-Uutela et al., 2021). Manure and not pretreated animal byproducts are however not allowed for insects as well as for any other animals (Leib et al., 2016). Different scenario is instead present in Central and South America. In Mexico no specific feed ban are present, thereby food waste as well as manure can be used for raising insects (Mexico, 2008). Similarly in Brazil, although not formally allowed by the legislation, several companies have embraced the way to recover nutrients from animal byproduct, food waste and manure through the use of insects, pushing the government to update the legislation (Allegretti et al., 2017).

5.3 Insect frass as fertilizers

Although insect frass have shown a strong potential for being used as fertilizer in agriculture (Barragán-Fonseca et al., 2022; Beesigamukama, Subramanian, et al., 2022), their use may impact the stability of the soil-plant ecosystem. Whether improperly used, frass such as every other organic fertilizer might be responsible for introducing pathogens to the soil, becoming a potential risk for human, animal or plant health (Kracmarova et al., 2020; Van Looveren et al., 2022). Therefore, organic material should be opportunely stabilized before being applied to the soil.

To the best of the authors' knowledge, only European Union has a specific legislation for using insect frass as organic fertilizers (Regulation (EU) 1925/2021). According to this regulation, insect frass should be considered as every other kind of animal manure and therefore can be applied to soil only after undergoing sanitizing treatment (i.e., 60 min drying at 70°C). The aim of this treatment is to inactivate pathogenic microorganisms and degrade phytotoxic substances potentially present in the frass (Lopes et al., 2022; Poveda, 2021; Van Looveren et al., 2022). In all other countries, such as US and Canada, where no specific legislation for insect frass as fertilizer is present, the standard law for animal manure management and organic fertilizers applies.

5.4 Insects as feed

Legislation for the use of insects as feed for food producing animals and pets is getting quickly updated. An interesting example can be found in the European Union, where insects have been recently accepted as feed for pig and poultry (Regulation (EU) 1372/2021). On the other hand, legislation in other countries is less permissive, still limiting the use of insects as feed at aquaculture and pet sectors. In the following section, current legislation about the possibility of using insects as feed in several countries is discussed. A list of laws regulating it in different countries is offered in Table 13.2.

5.4.1 Europe

European Union has the strictest legislation concerning the use of insects as feed. Nowadays in Europe insects are allowed to be used in aquaculture and for pig, poultry, fur animals and pets. While no limitations were ever present for feeding insects to pets (except if they are whole, frozen or dried but not milled, Commission Directive 82/475/EEC), the feed ban rules introduced for contrasting the transmission of spongiform encephalopathies (Reg. (EC) 999/2001) did not allow the

TABLE 13.2 Legislative framework concerning the use of insects as feed and pet-food around the world.

Country	Legislation	References
Africa	Act 36/1947 (South Africa)	Niassy, Ekesi, Hendriks, and Haller-Barker (2018)
	KS 2711:2016 (Kenya)	Niassy et al. (2022)
	KS 2921:2020 (Kenya)	Niassy et al. (2022)
Europe	Reg. (EU) 893/2017	IPIFF (2022)
	Reg. (EU) 1372/2021	IPIFF (2022)
	Reg. (EU) 1925/2021	IPIFF (2022)
	Reg. (EU) 999/2001	IPIFF (2022)
	Commission directive 82/475/EEC	IPIFF (2022)
North America	Federal Food, Drug and Cosmetic Act (USA)	Lahteenmaki-Uutela et al. (2021)
	Federal Food Additives Act (USA)	Lahteenmaki-Uutela et al. (2017)
	Feed Act (Canada)	Sogari et al. (2019)
	Feed Regulations (Canada)	Alagappan, Rowland, Barwell, Cozzolino, et al. (2022); Alagappan, Rowland, Barwell, Mantilla, et al. (2022)
	Health of Animals Act (Canada)	Alagappan, Rowland, Barwell, Cozzolino, et al. (2022); Alagappan, Rowland, Barwell, Mantilla, et al. (2022)
	Health of Animals Regulations (Canada)	Alagappan, Rowland, Barwell, Cozzolino, et al. (2022); Alagappan, Rowland, Barwell, Mantilla, et al. (2022)
	Organic Product Regulations (Canada)	Alagappan, Rowland, Barwell, Cozzolino, et al. (2022); Alagappan, Rowland, Barwell, Mantilla, et al. (2022)
Central and South America	Ley Federal de Sanidad Animal (Mexico)	Lahteenmaki-Uutela et al. (2017)
	Reglamento de Sanidad Animal (Mexico)	Lahteenmaki-Uutela et al. (2017)
	Especificaciones de los alimentos para consumo animal (Mexico)	Lahteenmaki-Uutela et al. (2017)
	Resolution 6612/2018 (Chile)	Valdes et al. (2022)
Asia	Rule 2010-142 (North Korea)	Valdes et al. (2022)
	Administrative Measures for Feed and Feed Additives (China)	Sogari et al. (2019)
	Control of Livestock and Fish Feed Act (Republic of Korea)	Lee et al. (2022)
	Insect Industry Promotion and Support Act (Republic of Korea)	Lee et al. (2022)
	Act of Safety Assurance and Quality Improvement of Feed (Japan)	Lee et al. (2022)
	Animal Feed Control Act B.E.2558 (Thailand)	Alagappan, Rowland, Barwell, Cozzolino, et al. (2022); Alagappan, Rowland, Barwell, Mantilla, et al. (2022)
	Feed Act 2009 (Malaysia)	Said and Bae (2019)
	Destruction of Disease-Bearing Insects Act 1975 (Malaysia)	Said and Bae (2019)
Australia	APVMA Good Manufacturing Practice	Lahteenmaki-Uutela et al. (2017)
	FIAAA Code of Practice	Lahteenmaki-Uutela et al. (2021)
	Australian FeedSafe Standard 5812-2017	Lahteenmaki-Uutela et al. (2021)

use of any animal protein in livestock nutrition. The regulation that opened the way for the use of insects as feed in Europe has been the Reg. (EU) 893/2017, which allowed seven species (*H. illucens*, *M. domestica*, *T. molitor*, *A. diaperinus*, *A. domesticus*, *G. sigillatus*, *G. assimilis*) for being used as feed in aquaculture. Since 2021, the same species have been then authorized for swine, poultry and fur animals nutrition as well (Reg. (EU) 1372/2021), while a new insect species (*B. mori*) has been introduced afterwards (Reg. (EU) 1925/2021). It should be noted that all of these regulations are valid for insect protein, while fat as well as live insects have never been banned (Lee et al., 2022) except for ruminants. Insects are not included in the scope of Reg. (EU) 1069/2009 about the health rules of animal byproducts not intended for human consumption, therefore the use of live insect in animal feed remains at the discretion of the EU Member States (IPIFF, 2022). On the other hand, whole (dried or frozen not milled) insects have not yet been allowed for any animals, although national authorities have the right for allowing such products as feed for not-food production animals, provided that there is no risk for animal health (IPIFF, 2022). However, regardless of the status of the product (authorized or not authorized), all the material used as feed should not represent a safety issue for the animals (Reg. (EC) 767/2009; Commission directive 2002/32). Specific regulation is present for food-producing animals should respect the standards defined in Reg. (EC) 1831/2003 about requisites for feed hygiene. It implicates that all the insect producers should register as ‘feed business operator’ and follow a series of feed safety rules.

5.4.2 Africa

In most of the African countries, no clear legislation about the use of insects and products thereof as animal feed is present (Lee et al., 2022). Most of these countries rely on international frameworks (e.g., from FAO and WHO) allowing an indiscriminate use of insects for feeding animals as long as they are safe for animals and humans (Niassy et al., 2018). In some cases, old and imprecise legislation limiting the use of animal proteins in animal nutrition may hinder the use of edible insects in the feed sector (Usman & Yusuf, 2021). For example in South Africa, the Act 36 of 1947 does not explicitly consider the use of insects as feed, but it emphasizes that all the feed should be safe for animals and humans (Niassy et al., 2022). High safety standards are also the characteristics needed for using insects as feed in Kenya (Niassy et al., 2022).

5.4.3 North America

In USA, the Association of American Feed Control (AAFCO) jointly with the Food and Drug Administration (FDA) are the two legal organisms regulating the feed sector (Lee et al., 2022). According to the current legislation, all the feed admitted to be used in United States should be GRAS (generally recognized as safe). If the material is not granted GRAS status, it should be considered as feed additives and follow the Food Additives Act (Lahteenmaki-Uutela et al., 2017). At the date, only *H. illucens* (as meal or whole dried larvae) has been granted with the GRAS status and is allowed for being used in fish, poultry, swine and wild bird rearing, while BSF oil has been recognized as safe for swine but it is still waiting for full approval by AAFCO (S. Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022; Lahteenmaki-Uutela et al., 2021). Concerning the use of insects as petfood, no AAFCO approval is requested (Lee et al., 2022). A status-specific legislation is therefore applied, with some US states already allowing insects for pet nutrition and other states waiting for acceptance from the federal government (Lahteenmaki-Uutela et al., 2021).

In Canada, while no restriction is present for insects as pet food (Valdes et al., 2022), insects in livestock nutrition are considered novel feed and therefore they should be evaluated for their safety before being authorized as feed (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022). The competent organism is the Canadian Food Inspection Agency (CFIA). At the date, only BSF larvae have been authorized for aquaculture and poultry feed (Lahteenmaki-Uutela et al., 2021).

5.4.4 Central and South America

Use of insects as feed is not strictly regulated in Latin America. Mexico, though permits the use of insects as feed, does not have specific regulations about it (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022), while Brazil tends to follow the Codex Alimentarius (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022), not considering yet insects as feed. However, Brazilian government allows the use of *H. illucens* meal in poultry nutrition (Lahteenmaki-Uutela et al., 2021). In Chile, use of insects for pets and food production animals is allowed as long as the products are not harmful for the animals and their nutritional value is guaranteed (Valdes et al., 2022).

5.4.5 Asia

Contradictory rules apply to the insects as feed sector in Asia. In China there are no specific limitations about insects as feed and general feed legislations apply. However, according to such legislation, new feed and feed additives need to be authorized by the Agriculture Administrative Department of the State Council and inserted in the *Feed Material Catalogue* (Lee et al., 2022). Similarly, several insect species such as mealworm, BSF, grasshopper, cricket and mosquito are allowed as animal feed in South Korea and Thailand, where however, this material should comply with the general feed legislation (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022; Sogari et al., 2019; Valdes et al., 2022). Slightly stricter law is present in Japan, where insects as feed, although allowed, should always be authorized by the Ministry of Agriculture, Forestry and Fisheries (Lee et al., 2022), while in North Korea and Malaysia, old legislations that consider insects as pests and/or forbid animal proteins in feed, prevent insects from being used as feed (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022; Valdes et al., 2022).

5.4.6 Australia

Insects as feed in Australia are regulated by both central and single states government. According to the central legislation, regular feed does not need authorization as long as it is only intended for nutrition purposes, is part of the normal diet of the animal, is not dangerous for the animal and does not contain drugs or active components (Lee et al., 2022). On the other hand, whether the feed is treated with drugs or it is intended as additive, a special authorization should be granted by the Australian Pesticide and Veterinary Medicine Authority (Lahteenmaki-Uutela et al., 2017). At the date, three insect species, namely *A. domesticus*, *Z. morio* and *T. molitor*, have been identified as feed in Australia (Valdes et al., 2022). Their use is strictly dependent on the state legislation. Indeed, while all the Australian states accept insects as feed in aquaculture, not all of them allow their use for poultry nutrition. Specifically, Queensland and South Australia restricted the use of insects for poultry, while in the Northern Territory use of insects in ruminants and pig diets is only allowed if they are reared on substrates free of materials of mammal and poultry origin (Alagappan, Rowland, Barwell, Cozzolino, et al., 2022; Alagappan, Rowland, Barwell, Mantilla, et al., 2022). Nevertheless, despite these exceptions, insects cannot usually be used for ruminant nutrition in Australia, while raw insects (live or untreated) are not allowed for feed purposes, irrespective of the animals to be fed (Lahteenmaki-Uutela et al., 2021).

Concerning the pet-food sector, no legislation is present in Australia, where the use of insects is regulated with voluntary industry standards defined by the Pet Food Industry Association of Australia (PFIAA) (Lahteenmaki-Uutela et al., 2021).

6. Conclusions and future recommendations

Insects have been traditionally harvested and consumed in many food cultures; however, due to concerns over climate change and food system sustainability, the industrial production of insects and their use as food and feed is now gaining momentum around the globe. Depending on the insect species, edible insects have been found suitable as feed for aquaculture, poultry, livestock and pet animals. With growing interest in sustainable and alternative protein sources, it is expected that edible insects could play significant role in animal nutrition in the future. The bioconversion of agrifood waste with insects is receiving a growing interest among researchers and stakeholders. The application of insects for agrifood waste management presents an innovative and ecofriendly solution for improving resource efficiency and promoting a more circular food system. However, it is important to note that further research is needed to optimize their production, understand their nutritional composition and ensure safety in animal consumption. There are a number of further challenges, mainly related to contaminants, safety, legislation limits and animal welfare. The regulatory demands and lack of a stable and consistent set of regulations across international borders are major challenges for insect producers to market insect products at a global scale. In summary, while the edible insects possess several opportunities, there are still several challenges to overcome which can only be addressed through joint efforts from researchers, industry and regulatory bodies.

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A2. Protein from insects—a new biosphere of opportunity

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Protein from insects—a new biosphere of opportunity

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9.1 Introduction

Edible insects have been proposed as high-value alternative protein sources for the future, exhibiting high-quality nutrient compositions along with potentially health-promoting constituents. Traditionally, insects are consumed in many parts of the world by some 2 billion people worldwide with more than 2100 insect species in the world known to be edible (Jongema, 2017). Edible insects have been identified as an important protein source, with a protein content that can reach a value of 77% on a dry basis, depending on the species (Rumpold & Schlüter, 2013a). Traditional consumption of insects predominantly relies on collection from their natural habitat. However, with an increasing demand for insect products, commercial rearing of insects is needed to ensure steady supplies and quality. In principle, insects can be reared on food waste or low-value substrates, which provides an attractive key for bringing the losses back to the food chain. Insects offer good food conversion ratios and require significantly fewer inputs in the form of land, fresh water and feed compared to traditional livestock systems. However, optimization of insect rearing broadly depends on the composition and consistency of insect feed, selection of insect species, technological parameters of rearing and regulatory guidelines (Ojha, Bußler, & Schlüter, 2020).

According to several global market insights, the global edible insects market is expected to rise in the next decade, owing to increasing protein demand, climate policies, changing

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dietary needs and ongoing innovation in insect-based products (Mancini et al., 2022). The recent approval of yellow mealworms and migratory locusts for human consumption in the EU is surely an indication of the increasing popularity of insect-based products in the Western population. This chapter outlines the important aspects of the edible insects' sector including factors affecting insect rearing, setup of an insect farm, insect processing, nutritional aspects of insects, opportunities and the current challenges faced by the insect industry.

9.2 Insect rearing

Insect rearing is based on the same concepts used for rearing other animals, although some differences need to be considered. Firstly, insects are poikilothermic animals meaning that slight differences in the rearing conditions might result in important differences in growth performances, nutritional and microbial composition. Moreover, their life cycle comprises different stages, whereby different environmental conditions may be required along their life cycle. Finding the right combination between environment, feed, and organism performances is the key point for rearing insects (Kok, 2021b). For example, the amino acid pattern of the insect proteins is not affected by insect diet, as observed for black soldier fly prepupae and tobacco hornworm larvae (Landry, Defoliart, & Sunde, 1986; Wang, Wu, Li, & Zhang, 2020); whereas, it is depended on the life stage in the case of holometabolous insects, like *Tenebrio molitor* and *Musca domestica* (Finke, 2002; Pieterse & Pretorius, 2013).

9.2.1 Rearing control parameters

Among the environmental parameters, temperature, relative humidity, and light exposure are the most important factors affecting insect growth, nutritional quality, and safety of the raised insects. Generally, higher temperature leads to shorter development time at the cost of increasing food consumption. Such phenomenon has been observed in *Schistocerca gregaria* reared at 25°C and 30°C (Akman Gündüz & Gülel, 2002) and *Hermetia illucens* raised at 20°C and 27°C, which is due to the higher metabolic rate in insects reared at higher temperature (Gligorescu, Toft, Hauggaard-Nielsen, Axelsen, & Nielsen, 2018). Similarly, the development of house cricket (*Acheta domesticus*) was faster and food consumption was higher at 35°C than at 25°C (Roe, Clifford, & Woodring, 1980). However, in all cases the final insect weight did not show significant differences, indicating that, while higher rearing temperature may reduce the rearing duration, it cannot be justified by higher production yield. Higher temperatures may also lead to a reduction in survival as showed in *H. illucens* larvae, which developed slightly faster at 33°C than at 27°C, although their mortality was almost triple (Raimondi et al., 2020). Further, reproductive performances could be also affected by high temperatures. For example, the grasshopper *Chrotogonus trachypterus* showed higher fertility by increasing the temperature from 20°C to 35°C, with a rapidly decreasing trend at temperatures higher than 35°C (Meena, Kumari, & Singh, 2020). Higher temperatures also led to shorter oviposition period and lower hatching ratio in grasshoppers and crickets (Meena et al., 2020; Ssepuuya et al., 2021).

Similar to the temperature, environmental humidity may impact insect growth performance. For example, relative humidity lower than 20% or higher than 80% reduced egg hatching and increased egg development time in *Dendrolimus tabulaeformis* (Han, Parajulee, He, & Ge, 2008). Larval performances were also affected by the relative humidity, with lower larval mortality and development time recorded at 60%, while higher body mass was obtained when 80/100% of relative humidity was set (Han et al., 2008). Differences connected to the relative humidity were also observed in other species such as *H. illucens*, which displayed higher adult emergence, higher eggs eclosion, lower development time and higher adult survival with 70% of relative humidity, rather than 25% or 40% (Holmes, Vanlaerhoven, & Tomberlin, 2012). However, despite these data, relative humidity has lower importance than temperature (Kotsou, Rumbos, Baliota, Gourgouta, & Athanassiou, 2021), with significant effects observed only when very high or very low humidity is set.

Concerning the photoperiod, its effect has not been deeply investigated so far, with few studies showing no differences in larval development time and survival, when different photoperiod conditions were tested (Savvidou & Bell, 1994). However, the effect of light exposition can vary depending on the insect species considered. For example, longer development time and higher final larval weight were observed for sawfly (*Neodoptrion sertifer*) reared at longer light exposition (Kollberg, Bylund, Schmidt, Gershenson, & Bjorkman, 2013). The intensity of the light also plays an important role. For example, Schneider (2020) noted better mating of *H. illucens* adults when exposed to wavelengths close to 450 or 550 nm.

Photoperiod and light intensity, as well as temperature and relative humidity, may also affect the nutritional composition of insects (Oonincx & Finke, 2021). For example, exposition at UV-B radius stimulated the production of vitamin D3 in *A. domesticus* (Oonincx et al., 2018), while lower temperature led to higher unsaturation levels in fatty acids of the mosquito *Culex tarsalis* and the cricket *Gryllus bimaculatus* (Harwood & Takata, 1965; Hoffmann, 1973).

Another critical factor showing a high impact on growth performances as well as the nutritional composition of the reared insects is the rearing density (Dobermann, Swift, & Field, 2017; Ortiz et al., 2016; Ribeiro, Abelho, & Costa, 2018). Higher population densities usually lead to poor food conversion efficiency, food consumption, individual weight, development time and survival (Barragan-Fonseca, Dicke, & van Loon, 2018; Dzepe, Nana, Fotso, Tchuinkam, & Djouaka, 2020; Grau, Vilcinskis, & Joop, 2017; Morales-Ramos & Rojas, 2015). It is usually due to competition between different subjects (Weaver & Mcfarlane, 1990), cannibalism (Howell & Clift, 1972; Ribeiro et al., 2018) and a higher risk of pathogens proliferation (Maciel-Vergara, Jensen, Lecocq, & Eilenberg, 2021). Crowding conditions have also shown negative effects on the reproductive performances of adult insects, leading to smaller insect size and consequently lower fecundity (Morales-Ramos, Rojas, Kay, Shapiro-Ilan, & Tedders, 2012; Pastor et al., 2011; Peters & Barbosa, 1977) or eggs cannibalism (Kuriwada, Kumano, Shiromoto, & Haracuchi, 2009; Longstaff, 1995). Despite this, lower densities are not always favorable because they can lead to lower production yields (Barragan-Fonseca et al., 2018), making the rearing process not profitable in the long period and requiring more space for producing the same amount of insects (Ortiz et al., 2016). Furthermore, some insect species, such as *H. illucens* showed better nutrient utilization and growing performances when higher densities were adopted

(Barragan-Fonseca, Dicke, & van Loon, 2017). High rearing density can also allow higher mating frequency, lower genetic stagnation, higher mass of produced eggs, and higher egg hatchability due to the warmer and moister microenvironment in the oviposition medium (Jensen, Kristensen, Heckmann, & Sørensen, 2017; Peters & Barbosa, 1977).

Another element that influences insect performance in commercial rearing is the feed. Choice of the most suitable rearing substrate should consider several aspects as nutritional requirements of the insect species, insect ability to grow on that diet, nutritional properties and safety of the final products. Acceptability and growing performances mainly depend on the palatability and nutritional composition of the substrate (Adebayo, Kemabonta, Ogbogu, Elechi, & Obe, 2021; Fowles & Nansen, 2020; Morales-Ramos, Rojas, Dossey, & Berhow, 2020; Singh, Marathe, Raghunathan, & Kumari, 2021). For example, black soldier fly larvae grow faster in hen feed, maize distilled and okara diets than in brewer's grains (Galassi et al., 2021). Similarly, *Alphitobius diaperinus* larvae growth better on substrate richer in yeast, where higher weight, faster development and higher survival were noticed (Kotsou et al., 2021). Nevertheless, the best substrates in terms of growth are not always the best in terms of nutritional profiles.

9.2.2 Setting up an insect farm

Insect rearing can be performed with complete domestication (all the life cycle phases are run in the farm) or partial domestication (young insects are harvested in the wild and raised in the farm) (Guiné, Correia, Coelho, & Costa, 2021). If the full rearing is performed within the farm, several structures for housing insects should be planned (Kok, 2021a). One example of such rearing has been reported by Sheppard, Tomberlin, Joyce, Kiser, and Sumner (2002). In this procedure, black soldier fly eggs were laid on corrugated cardboard and incubated at 30°C for hatching. Neonate larvae were moved to a starter medium and let feed for 2–3 days, then transferred in a 5-L bucket where 500 larvae were fed every day up to the prepupae stage. Afterwards, the emerged adults were released in a rearing cage and let mate and lay eggs (Fig. 9.1). Although this is a very simple and rudimentary rearing procedure, it can already explain the complexity of insect farm management. Indeed the scope of the aforementioned method was just to use BSF (Black Soldier Fly) for manure management. When food/feed production is the goal, two rearing lines, that is the breeding line and the production line (Fig. 9.2), should be planned (Jensen et al., 2017). In the breeding line, adult flies are the main actors, while eggs are the main products. These should feed the production line and at the same time give new adults for renewing the cycle. Along such line, genetic selection and selective breeding might be performed with the scope of enhancing fertility, survival, development time, feed conversion, nutritional profile, disease resistance, etc. (Eriksson & Picard, 2021; Jensen et al., 2017). On the other hand, the production line is where insects are raised for food or feed utilization. Therefore, the final products of this line must be safe for the consumers and characterized by a high nutritional profile.

Production and reproduction lines can coexist in the same farm or be separated into two different farms. In the latter option, the rearing farm purchases eggs or young larvae from the reproducing farm and sells mature larvae or adults ready to be consumed or further processed. In both cases, the aim of commercial rearing is to generate income by

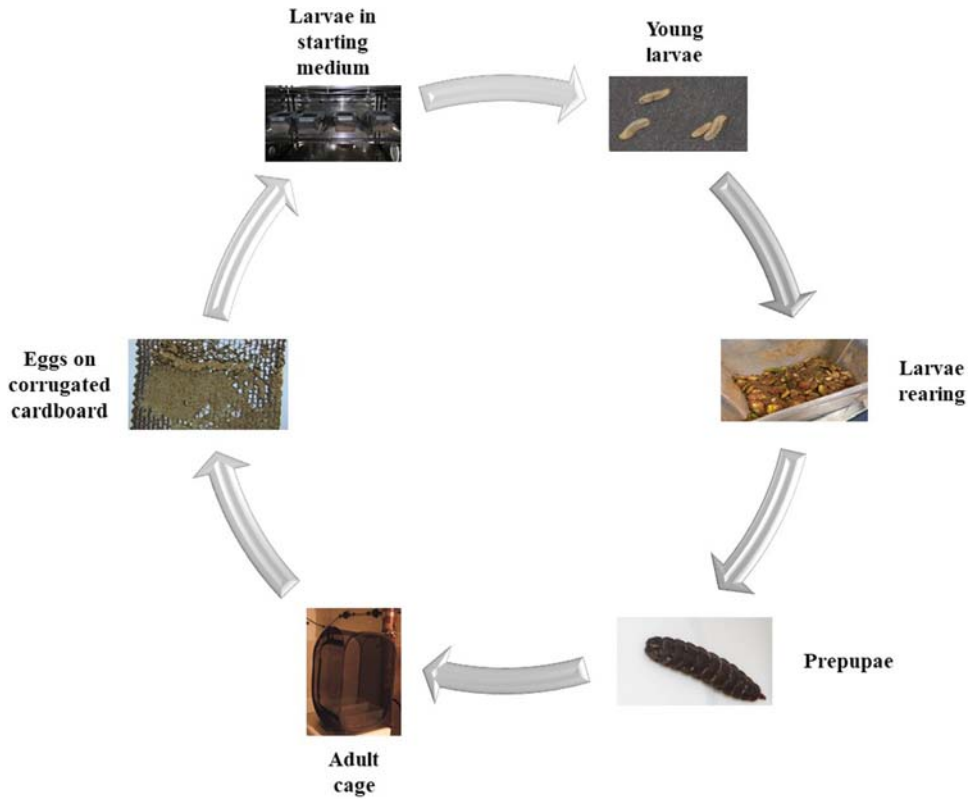


FIGURE 9.1 Black soldier flies rearing procedure. Source: Adapted from Sheppard, D. C., Tomberlin, J. K., Joyce, J. A., Kiser, B. C., & Sumner, S. M. (2002). Rearing methods for the black soldier fly (Diptera: Stratiomyidae). *Journal of Medical Entomology*, 39(4), 695–698. <https://doi.org/10.1603/0022-2585-39.4.695>.

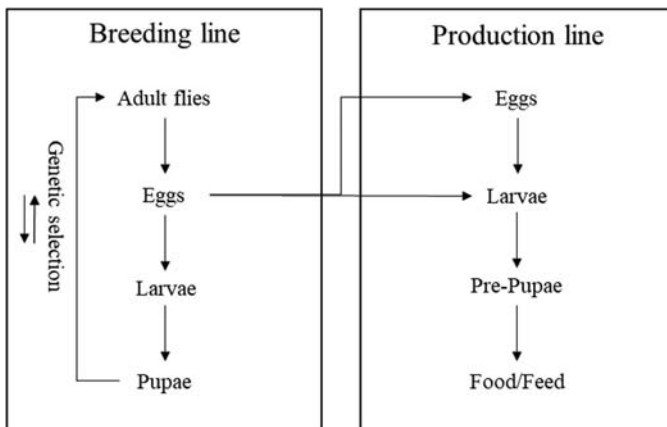


FIGURE 9.2 Example of insect rearing structures: breeding line and production line.

maximizing outputs (e.g., produced insects) and minimizing inputs (e.g., energy, labor, feed, water) and system leakage (e.g., dead insects). An overall and component-based mass balance approach should then be applied (Kok, 2021a) and automation should be implemented along the rearing line. Breeding synchronization and setting of several rearing units, as well as organization of the rearing process in several sub-processes (Kok, 2021c; Tomberlin, Sheppard, & Joyce, 2002), are the keys for such implementation. Furthermore, computerized control of the insect state, their biological performances, nutritional profile and feed requirements may help the entrepreneur to take an instantaneous decision (Ortiz et al., 2016), minimizing the risk of pathogens spreading (Joosten et al., 2020), building adequate feed rations for each development stage (Jensen et al., 2017) and creating an ideal environment for each insect population (Kok, 2021b).

9.3 Processing of insects

In the western world, there is consumer disapproval of insects due to their appearance (Chen, Feng, & Chen, 2009). Considering, that western countries did not develop entomophagy (van Huis, 2016), insects will be more likely consumed when invisible (Schösler, De Boer, & Boersema, 2012). Insects processed in a meal or dried powder present an attractive choice to be incorporated into regular foods. Furthermore, there is a need to develop tailored, highly nutritional products with a controlled composition (Purschke, Tanzmeister, et al., 2018). The production of protein-rich flour from insects for incorporation into foods provides an opportunity, for insect protein to achieve widespread exploitation in both food and feed. Therefore, there is a growing interest in generating protein isolates and concentrates (Ndiritu, Kinyuru, Kenji, & Gichuhi, 2017).

9.3.1 Solid–liquid extraction

Insect proteins are extracted in an aqueous or alkaline solution, as reported for *T. molitor*, *Zophobas morio*, *A. diaperinus*, *A. domesticus*, and *Blaptica dubia* (Ndiritu et al., 2017; Yi et al., 2013) or *T. molitor*, *A. domesticus*, *H. illucens*, and *Logusta migratoria* (Laroche et al., 2019; Mintah et al., 2020; Purschke, Tanzmeister, et al., 2018; Zhao, Vázquez-Gutiérrez, Johansson, Landberg, & Langton, 2016), respectively. Afterwards, proteins are isolated via pH adjustment and precipitation at their isoelectric point, followed by phase separation and drying, as demonstrated for *T. molitor*, *A. domesticus*, and *H. illucens* (Laroche et al., 2019; Mintah et al., 2020; Zhao et al., 2016). Prior to extraction, insects are often defatted, which has been shown to positively affect the protein extraction yield (Choi, Wong, & Auh, 2017) and purity (Laroche et al., 2019) from mealworm, cricket and silkworm pupae. Other factors that can influence the protein yield are pH of the solvent, extraction temperature, ratio of solvent to substrate, extraction time and salt concentration. For instance, it has been demonstrated for black soldier fly larvae and mealworm larvae, that protein extraction can be enhanced with an alkaline, or strongly acidic solvent, mild heating, higher volume of solvent in respect to the insect substrate, longer extraction time and presence of NaCl (Mintah et al., 2020; Yi, Van Boekel, & Lakemond, 2017; Zhao et al., 2016). Finally, the extractability of proteins can be

improved by the implementation of emerging food processing technologies (Ojha, Bußler, Psarianos, Rossi, & Schlüter, 2021). This has been confirmed after sonication of *S. gregaria*, *Apis mellifera*, *T. molitor*, *G. bimaculatus*, and *Bombyx mori* (Choi et al., 2017; Mishyna, Martinez, Chen, & Benjamin, 2019) and pulsed electric fields (PEFs) treatment of *A. Domesticus* (Psarianos et al., 2021).

The defatting step, prior to protein extraction, has been suggested to be crucial for protein quality, as reported for *G. bimaculatus* (Jeong, Lee, & Cho, 2021). The method, solvent and duration of defatting have been shown to affect the efficiency of the extraction from crickets and mealworms (Laroche et al., 2019; Zhao et al., 2016). However, the solvent used for defatting can affect the functional properties of the proteins, including solubility, oil and water binding capacity, emulsifying capacity, foaming properties and gelation, as well as protein secondary structure of proteins obtained from *G. bimaculatus* (Jeong et al., 2021).

The conditions of protein extraction have been shown to affect the quality of the proteins, as in the case of black soldier fly larvae (Mintah et al., 2020). Temperature, NaCl concentration and pH can influence solubility (Bußler, Rumpold, Jander, Rawel, & Schlüter, 2016; Udomsil, Imsoonthornruksa, Gosalawit, & Ketudat-Cairns, 2019) and fluorescence intensity (Bußler et al., 2016) of proteins from crickets and mealworm and black soldier fly larvae. Proteins extracted in a saline solution show higher emulsifying and foaming capacity and stability than water-soluble proteins (Kim et al., 2019). Additionally, pH and salt concentration can affect the rheological properties of protein extracts from *T. molitor* (Zhao et al., 2016), as well as the color of the protein fractions from *T. molitor*, *Allomyrina dichotoma*, and *Protaetia brevitarsis seulensis*, due to enzymatic browning (Kim et al., 2019; Yi et al., 2017). A further effect of the protein extraction conditions on protein quality was observed in the variation of the amino acid composition (Kim et al., 2019; Mintah et al., 2020) and molecular weight (Kim et al., 2019; Yi et al., 2017) of the isolated proteins from *T. molitor*, *H. illucens*, *Allomyrina dichotoma*, and *P. seulensis* with different extraction conditions.

9.3.2 Dry fractionation

Edible insects have been identified as strong candidates for dry fractionation due to their composition and protein quality. A variety of preprocessing steps of the insects has been suggested, such as deshelling, microbial and enzymatic stabilization, defatting, drying and milling (Sweers et al., 2022). Milling is a crucial step for efficient dry fractionation (Pelgrom, Vissers, Boom, & Schutyser, 2013). Commonly, dry fractionation can be performed with sieving, air classification and electrostatic separation (Assatory, Vitelli, Rajabzadeh, & Legge, 2019; Schutyser & Van der Goot, 2011). The protein content can significantly differ in the various generated fractions, depending on the particle size, drying method, possible defatting and thermal pretreatment, as shown for fractions from mealworm larvae (Purschke, Brüggén, Scheibelberger, & Jäger, 2018), while the amino acid content and solubility of different fractions can vary, as reported for mealworms and crickets (Sipponen et al., 2018). The highest protein recovery from dry fractionation has been reported for fractions of medium particle size, as in the case of mealworm larvae (Purschke, Brüggén, et al., 2018). Nevertheless, there is only a very limited number of studies on the dry fractionation of insects and it is a field that requires further investigation.

9.3.3 Enzymatic

Protein hydrolysate is a fraction containing amino acids, peptides and oligopeptides, which is generated by partial protein hydrolysis (Clare & Swaisgood, 2000). The hydrolysis of insect proteins aims to generate bioactive peptides or enhance the functional properties of the insect proteins (da Silva Lucas, de Oliveira, da Rocha, & Prentice, 2020; Nongonierma & FitzGerald, 2017). As reported for several species, including locusts, lesser mealworms and crickets, initially insects are homogenized with water and temperature and pH are adjusted to the desired values for the targeted enzymatic activity. The enzyme is added to the mixture that is incubated for a specific amount of time. The enzymatic treatment is followed by enzyme inactivation and recovery of the protein hydrolysate (Batish et al., 2020; Leni, Soetemans, Caligiani, Sforza, & Bastiaens, 2020; Liceaga, 2019; Purschke, Meinschmidt, Horn, Rieder, & Jäger, 2018).

The degree of hydrolysis is influenced by several factors, including the selection of enzyme, ratio of enzyme to substrate, duration, pH and pretreatment (Purschke, Meinschmidt, et al., 2018). It can affect the functional properties of the proteins including solubility, water and oil binding capacity and emulsifying and foaming properties, as demonstrated for *Locusta migratoria* (Purschke, Meinschmidt, et al., 2018), *A. diaperinus* (Leni et al., 2020), and *Gryllodes sigillatus* (Hall, Jones, O'Haire, & Liceaga, 2017). Regarding bioactive peptides, the degree of hydrolysis can influence their activity (Najafian & Babji, 2012), including enzyme inhibition and antioxidant activity, as shown for *G. sigillatus* (Hall, Johnson, & Liceaga, 2018) and *A. diaperinus* (Sousa, Borges, & Pintado, 2020). Finally, a thermal treatment prior to the enzymatic treatment has been demonstrated to affect the bioactivity of *G. sigillatus*, *T. molitor* and *S. gregaria* (Zielińska, Baraniak, & Karaś, 2017).

9.4 Nutritional aspects—novel bioactives from insects

Edible insects have a rich nutritional profile with a high protein and fat content (Rumpold & Schluter, 2013a, 2013b). The lipid fraction is of high quality containing fatty acids generally comparable to those of poultry and fish in their degree of unsaturation, but insects contain higher levels of polyunsaturated fatty acids. Insect proteins contain both essential and nonessential amino acids, as they have been identified in insect matrices, such as *Macrotermes nigeriensis*, *Gryllus assimilis*, *Melanoplus foedus*, and *Cirina forda* (Oibiokpa, Akanya, Jigam, Saidu, & Egwim, 2018). Additionally, they are a potential source of biologically active compounds that have health benefits beyond simple nutritional values (Mlcek, Borkovcova, Rop, & Bednarova, 2014).

9.4.1 Bioactive peptides

Peptides that are considered bioactive are the ones that show a biological function such as antimicrobial, antioxidant or immune modulatory (Lemes et al., 2016). The presence of bioactive peptides in insects has been suggested for several species including mealworm, house cricket, silkworm, and leafworm among others (da Silva Lucas et al., 2020).

9.4.1.1 Antioxidant peptides

Insects, after being subjected to partial hydrolysis, have been identified as a source of antioxidant peptides. In specific, hydrolysates obtained from in vitro digestion of cockroach, locust, superworm, cricket, and mealworm species have been shown to have antioxidant activity. This antioxidant activity has been evaluated with different methods, including free radical scavenging, chelating and reducing power (Messina et al., 2019; Zielińska, Karaś, & Jakubczyk, 2017). The antioxidant activity of these peptides can be enhanced with a higher degree of hydrolysis and a lower molecular weight (Hall et al., 2018; Yang et al., 2013) since low molecular weight peptides have more amino acids exposed to interact with free radicals (Hall et al., 2017). Nevertheless, it has also been suggested that the antioxidant activity of these peptides is depended on the insect species and not on the degree of hydrolysis (Zielińska, Karaś, et al., 2017). Furthermore, the amino acid pattern and the sequence of these peptides play an important role in their activity (Sarmadi & Ismail, 2010).

9.4.1.2 Inhibitory peptides

Some of the bioactive peptides, found in edible insects, are able to assist and module the immune system, since they have an inhibitory effect on enzymes related to several diseases (da Silva Lucas et al., 2020).

In specific, hydrolysates obtained from crickets after simulated gastrointestinal digestion can inhibit dipeptidyl peptidase-IV (DPP-IV), which is linked to the development of diabetes (F. Hall et al., 2018). Protein hydrolysates obtained from yellow mealworm after enzymatic treatment has been shown a significant DPP-IV inhibition (Dávalos Terán, Imai, Lacroix, Fogliano, & Udenigwe, 2020), while a combination of ultrasound and enzymatic treatment of yellow mealworm can generate peptides that can inhibit α -glucosidase to help combat diabetes (Rivero-Pino, Espejo-Carpio, Pérez-Gálvez, Guadix, & Guadix, 2020).

Additionally, insect-derived hydrolysates can inhibit angiotensin-I converting enzyme (ACE), thus contributing to battling hypertension (Cito, Botta, Francardi, & Dreassi, 2017). The ACE-inhibiting peptides have been identified and evaluated in vivo and in vitro from several different species, such as *T. molitor* (Dai, Ma, Luo, & Yin, 2013), *Drosophila melanogaster* (Staljanssens et al., 2011), *Bombyx mori*, *S. gregaria*, and *Bombus terrestris* (Vercruysse, Smagghe, Herregods, & Van Camp, 2005) among others.

The inhibition capacity of these peptides is correlated to the degree of hydrolysis (da Silva Lucas et al., 2020), as well as the sequence of the C-terminus of the peptide, since ACE, for instance, is reported to interact with hydrophobic C-terminal (Neves et al., 2017).

9.4.1.3 Antimicrobial peptides

Antimicrobial peptides are usually small, cationic and heat-resistant peptides that can form ion channels or transmembrane pores on the cell membrane of microorganisms, thus inactivating them (Park & Hahm, 2005). Their antimicrobial capacity is affected by the lipid composition and electric potential of the cell membrane, since they tend to bind on phospholipids and phosphate groups of the cell membrane (Veldkamp, Dong, Paul, & Govers, 2021). Their main categories are α -helical peptides, cysteine-rich peptides and proline-rich peptides, while there are different mechanisms of the permeabilization of cell membranes by antimicrobial

peptides (Duclohier, 2002; Veldkamp et al., 2021). Furthermore, they can be utilized synergistically with antibiotics (Rossi, Rangasamy, Zhang, Qiu, & Wu, 2008). Antimicrobial peptides have been identified in edible insects and show a variety based on the insect species (de Castro, Ohara, dos Santos Aguilar, & Domingues, 2018; Rahnamaeian et al., 2015).

9.4.2 Chitin and chitosan

Chitin is a polysaccharide that is the main constituent of the exoskeleton of invertebrates and can be found in fungi and yeasts, as well. Chitin has been identified, isolated and characterized from a wide variety of insect species. It is usually isolated via sequential purification steps that aim to remove other substances of the insect material, such as lipids, proteins and minerals (Mohan et al., 2020). A lack of chitinase can make chitin indigestible, thus functioning as a dietary fiber (Muzzarelli et al., 2012), while the presence of chitinase can make chitin digestible, thus functioning as an energy source (Mlcek et al., 2014). Chitosan is the product of the deacetylation of chitin (Muzzarelli, 2013). Chitosan offers a variety of medicinal applications (Morin-Crini, Lichtfouse, Torri, & Crini, 2019). Insect-derived chitosan is being underlined for its antimicrobial, antitumor, antiviral and antioxidant activity, as in the case of housefly larvae, cockroaches and mealworms (Ai, Wang, Xia, Chen, & Lei, 2012; Chen et al., 2021; Ibañez-Peinado, Ubeda-Manzanaro, Martínez, & Rodrigo, 2020).

9.4.3 Phenolic compounds

Insects have been shown to form phenolic compounds during their sclerotization process via enzymatic reactions (Andersen, 2010; Sugumaran, 2010). However, their phenolic composition has, also, been linked to their dietary habits and is attributed to them being herbivorous (Nino, Reddivari, Osorio, Kaplan, & Liceaga, 2021). Phenolic compounds are considered important bioactive due to their antioxidant activity (Zheng & Wang, 2001). However, insect-derived phenolics have, also, shown the potential for antiinflammatory, antitumor and antimicrobial activity (Nino, Reddivari, Osorio, et al., 2021). Total phenolic content, flavonoid content, tannin content, but also antioxidant activity, have been estimated in extracts from several insects species including *A. dosmesticus*, *T. molitor*, *Z. morio*, *Rhynchophorus ferrugineus*, *Rondotia menciana*, and *Holotrichia parallela* (Botella-Martínez, Lucas-González, Pérez-Álvarez, Fernández-López, & Viuda-Martos, 2021; Hirayama, Ono, Meng, Shimada, & Daimon, 2013; Liu et al., 2012; Nino, Reddivari, Ferruzzi, & Liceaga, 2021).

9.4.4 Vitamins

Insects have been shown to contain vitamins A, B2, B6, B12, D, and E. Holometabolous insects contain low levels of vitamin A, while holometabolous larvae contain no retinoids. Vitamin E content in insects has a high variation depending on the species and has been observed to be higher in wild insects than in reared ones due to their diet (Oonincx & Finke, 2021). Vitamin D was observed mainly in wild insects (Finke, 2015), but has been, also, reported to be formed due to UVB exposure in crickets, locusts, mealworms, and black soldier fly larvae (Oonincx et al., 2018).

Insects have high potential as a source of bioactive compounds that can be beneficial to human health and even have medicinal applications. However, there is a limited number of studies focusing on these compounds and how they are affected by different processing and cooking methods or storage conditions. It is considered necessary that the bioactive components of insects are studied more thoroughly.

9.5 Challenges and opportunities

Although the use of insects as food for humans or feed for animals presents several benefits, proposing such “*unusual*” organisms as novel nutrients source may arise challenges. An overview of the main challenges and opportunities connected with insects as food and feed is shown in [Table 9.1](#)

Among the opportunities, food security and health-promoting capacity are the main aspects to consider. Besides these, sustainability and nutrient recycling capacity should always be addressed. Such issues are closely dependent on the reared species. Choosing the insect species is therefore the first matter to be addressed. Species belonging to the Coleoptera, Diptera and Orthoptera orders seem to be the most suitable for mass rearing and nutrient production. However, other species should be further exploited. The ability to grow on alternative substrates, nutritional profile, production yield, vulnerability to diseases, and consumers’ acceptability should be the main criteria leading to this choice ([Rumpold & Schluter, 2013b](#)).

Concerning the rearing medium, a good substrate should be able to satisfy all the needs of the insect species, guaranteeing fast growth, high production yield and suitable nutrient composition of the harvested insect ([Pinotti & Ottoboni, 2021](#)). Furthermore, it should be cheap, available throughout the year, and able to ensure the sustainability of the production.

TABLE 9.1 Challenges and opportunities of insects as food and feed.

Challenges	Opportunities
Species selection	Food security
Substrate selection	Human and animal health
Food safety	Environmental sustainability
Scaling-up	Circular economy
Rearing and processing optimization	Alternative farming methods (urban farming)
Production cost and product price	Technical innovation (3D printing)
Automation and mechanization	Smallholder livelihoods
Consumers acceptability	Pest control
International legislation uniformity and international trading	New food items (new flavors)
Insect welfare and diseases management	

Environmental sustainability is one of the most interesting opportunities connected to edible insects. In general, insects show lower GHG (Green House Gas) emissions, lower water and carbon footprints, lower land use and higher feed conversion ratio than conventional livestock (Guiné et al., 2021). However, such advantages are strictly dependent on the feed used for their rearing, as using conventional feed may result in lower environmental advantages than normally expected (Bosch & Swanson, 2021; Smetana, Spykman, & Heinz, 2021). Therefore, in order to explore such interesting potentiality and protect the environment, low-value materials such as waste and by-products should be used. From such a perspective, insects may perfectly fit within the circular economy concept (Smetana et al., 2021), playing a crucial role in nutrient recycling and not competing with humans and other animals for resource use (Gahukar, 2016).

Nevertheless, when insects are intended to be used as food for humans or feed for food-producing animals, food waste and manure are generally not allowed as rearing substrates (Lahteenmaki-Uutela, Marimuthu, & Meijer, 2021). The main reason for such a ban is related to hygienic and safety concerns. Indeed, pathogenic microbial species such as *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 have been detected in the gut of insects growth on waste and manure (Vandeweyer, De Smet, Van Looveren, & Van Campenhout, 2021), albeit over time reduction of such pathogens in the substrate and the insect gut was observed (Elhag et al., 2022; Van Moll et al., 2022). Accumulation of chemical hazards in tissues of insects raised on contaminated diets may also happen (Meyer, Meijer, Hoek-van den Hil, & Van der Fels-Klerx, 2021). The use of safe and currently allowed substrate and adequate processing techniques would minimize the safety risks of edible insects.

Different substrates as well as different post-harvest processes may also have a strong impact on the nutritional composition and nutrient bioavailability (Ojha et al., 2021; Oonincx & Finke, 2021), making an obstacle to industrialization of insects production. Indeed, in order to implement insects as human food or animal feed, standardization of their chemical composition should be pursued (Liland et al., 2021). Therefore, the definition of standard rearing and processing strategies is an important hurdle to overcome.

A further aspect that should be considered when insects are expected to be used as food is the presence of allergens and antinutrients. Several allergens, such as tropomyosin, arginine-kinase, paramyosin, and chitinase, have been described in insects and co-sensitization of people allergic to crustaceans and house dust mites has been observed (Ribeiro, Sousa-Pinto, Fonseca, Fonseca, & Cunha, 2021). Furthermore, people constantly exposed to insects (i.e., workers in insect farms) may be more susceptible to developing allergic reactions.

Among the challenges, consumers' acceptability should always be accounted for. Despite a strong tradition of insect consumption as a delicacy in several countries, there is still a strong repugnancy towards insects as food or feed in western societies (van Huis, 2015). It is mainly due to the perception that insects are dirty, unsafe and disgusting (Wassmann, Siegrist, & Hartmann, 2021), which may make the edible insects business not economically affordable. In order to overcome such limitations, communication strategies focusing on environmental and health benefits connected with insect consumption should be implemented. Increased market presence of insect-based products, higher consumption opportunities and lower selling prices may help to increase the willingness to buy edible

insects as well (Wassmann et al., 2021). Selling price is a particularly interesting and poorly considered topic. So far, edible insects are an expensive food, due to the high production cost connected to feed, energy input and labor (Niyonsaba, Hohler, Kooistra, Van Der Fels-Klerx, & Meuwissen, 2021). Therefore, Scaling-up and mechanization for fast manipulation, feeding and harvesting of the insects is much needed (Rossi, Bosch, Pisa, & Oonincx, 2022; Rumpold & Schluter, 2013b). Automated and not destructive systems for quality and safety monitoring should also be developed (Rossi, Durek, Ojha, & Schluter, 2021). However, scaling up may not always be desired. For example, in developing countries, insect production is mainly delegated to small farmers, who are just interested in the production of feed for their own animals and food for themselves (Chia, Tanga, van Loon, & Dicke, 2019). In such areas, harvesting of insects from the wild may be implemented, allowing to reach further advantages such as crop protection and control of pest outbreaks and plagues (van Huis, 2021a).

Further, in developed countries, scaling up production may not always be the right decision. Insects are the only livestock that can grow in crowded and artificial environments such as inside house kitchens. It means that they can perfectly fit within the urban farming concept so that in an ideal situation, every household has its own insect farm and is able to produce its food (Dunkel & Payne, 2016). In a simile overview, insects may also be able to grow in space, applying for becoming a future food item for space travel and space colonies (Kok & van Huis, 2021).

When insects are reared in high density in small spaces, concerns regarding animal welfare as well as the possibility of diseases spreading need to be considered. Insect diseases are a main hurdle for the producers since they can reduce insect fitness and productivity or cause insect death. However, insect pathogens have not been observed for showing pathogenicity in humans or other vertebrate animals so far (insects do not seem to be able to zoonosis transmission), albeit insects might carry human pathogens (Galecki & Sokol, 2019; Maciel-Vergara et al., 2021). Insect wellness is instead an aspect that should be carefully assessed. Two items should be considered, that is the ability of insects to experience pain and the high number of living beings to be killed. No clear results about the perception of pain in invertebrates have been drawn so far, whereby the precautionary principle should be applied (van Huis, 2021b). The concept of killing animals is a popular topic, which is responsible for vegetarian and vegan diets spreading. However, low consideration seems to be committed to insects since they are considered disgusting beings and are much less similar to humans than mammals and other common animals (van Huis, 2021b).

9.6 Conclusions

Insects are a nutrition-dense source of protein that has gained considerable worldwide popularity in the last decade. Several parameters including temperature, relative humidity, light exposure, population density and diet are important for optimum growth, nutritional quality and safety of the reared insects. Protein isolates and concentrates can be prepared by solid—liquid extraction, and dry fractionation of enzymatic hydrolysis. Emerging food processing technologies (e.g., sonication and PEF) can improve the extractability of

proteins while reducing or eliminating the usage of chemicals. Apart from proteins, insects can be a potential source of novel bioactive including bioactive peptides, chitin, phenolic and vitamins. While the insects possess several opportunities, there are still several challenges to overcome which can only be addressed through joint efforts from researchers, industry and regulatory bodies.

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A3. Edible insect processing pathways and implementation of emerging technology

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Edible insect processing pathways and implementation of emerging technologies

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REVIEW ARTICLE

Abstract

The processing of insects is paramount to deliver safe and high quality raw materials, ingredients and products for large-scale food and feed applications. Depending upon the nature of the initial material and the desired end product, the processing pathways vary and may include several unit operations currently already used in food and feed processing. Insect processing pathways can involve harvesting, pre-processing, decontamination, further processing, packaging and storage. Several traditional and industrial decontamination methods have been proposed for edible insects, which include smoking, drying, blanching/boiling, marination, cooking, steaming, toasting and their combinations. Further processing steps are employed to produce insect meal, insect flour or extracted insect fractions. Each operation will have a different impact on the chemical and microbiological properties of the final product. Novel food processing technologies (e.g. high pressure processing, pulsed electric field, ultrasound and cold plasma) have shown potential to modify, complement or replace the conventional processing steps in insect processing. These technologies have been tested for microbial decontamination, enzyme inactivation, drying and extraction. Further, these are considered to be environmentally friendly and may be implemented for versatile applications to improve the processing efficiency, safety and quality of insect based products. Future research focuses in insect processing are development of efficient, environmentally friendly and low-cost processes; waste minimisation and incorporation of by-products/co-products.

Keywords: pre-processing, nonthermal technologies, extraction, novel food, microbial decontamination

1. Introduction

Edible insects have been part of the human diet throughout history (Dobermann *et al.*, 2017). Their consumption is traditionally recognised in several countries, where they are predominantly collected from their natural habitat (Dobermann *et al.*, 2017); however, with increasing demand of insect based foods in western countries, many companies have started mass rearing systems (Wade, 2020).

In order to ensure food safety, insect processing and storage should follow the same health and hygiene standards as for any other traditional food or feed (Imathiu, 2020). Hazards which are commonly found in insects comprise pathogenic microorganisms and parasites (Gałęcki and Sokół, 2019; Schlüter *et al.*, 2017), allergens (De Gier and

Verhoeckx, 2018), heavy metals, toxins and other chemical contaminants (Bosch *et al.*, 2017; De Gier and Verhoeckx, 2018; Poma *et al.*, 2017, 2019; Van der Fels-Klerx *et al.*, 2016). Prions and human pathogenic virus should be considered as well (EFSA, 2015), although they have not been detected in edible insects yet.

Processing technology depends on insect species, safety hazards and type of final product. Edible insects can be marketed in three different forms: (1) whole (dried, frozen, pre-cooked); (2) processed; and (3) extracts (EFSA, 2015). Whole insects are commercially available as dried, frozen or chilled products for direct use by the consumer or food manufacturer. Processing of insects results in powder or paste, which allow the insects to be incorporated into food products or directly into dishes prepared by the consumer.

In order to improve the acceptance of insects and insect products and extend their shelf life, several traditional cooking techniques such as steaming, roasting, smoking, frying, stewing, curing (Ebenebe and Okpoko, 2015; Grabowski and Klein, 2017b; Lautenschläger *et al.*, 2017; Manditsera *et al.*, 2019; Nonaka, 2009; Obopile and Seeletso, 2013; Ramos-Rostro *et al.*, 2016; Shockley *et al.*, 2018) have been proposed. Other techniques, such as traditional sun-drying (Manditsera *et al.*, 2018), microwave processing (Lenaerts *et al.*, 2018; Vandeweyer *et al.*, 2017b), freeze-drying, oven-drying (Azzollini *et al.*, 2016; Fombong *et al.*, 2017), dry heat treatment (Bußler *et al.*, 2015), dry fractionation (Purschke *et al.*, 2018a), freezing (Melis *et al.*, 2018), marination, fermentation (Borremans *et al.*, 2018, 2020b; Patrignani *et al.*, 2020) and new processing methods as ultrasound-assisted extraction (Mishyna *et al.*, 2019; Panja, 2018; Sun *et al.*, 2018), cold atmospheric pressure plasma (CAPP) (Bußler *et al.*, 2016a), supercritical CO₂ extraction (Purschke *et al.*, 2017), enzymatic hydrolysis (Purschke *et al.*, 2018a) for protein, fat, and/or chitin extraction, three-dimensional food printing technologies (Severini *et al.*, 2018; Soares and Forkes, 2014), and several modified atmosphere packaging methods (Flekna *et al.*, 2017; Stoops *et al.*, 2017) have also been tested for insects and insect products.

Main objective of processing is to ensure food safety; however, it is also important to satisfy quality standards and consumer expectations. In this context, an important aspect to consider is the microbiological safety. In countries where insects are already recognised as food or feed, specific microbiological criteria are present. However, in the other countries, lack of specific rules may be observed. For example, insects are classified as novel foods in European Union (EU) and therefore fall within the current novel food legislation (EU Regulation 2283/2015; EFSA, 2016). Whole edible insects and their derived ingredients can only be lawfully placed on the EU market after safety assessments and authorisations. When a novel food is accepted for consumption, it will be considered as any other food, therefore it should respect the current accepted limit for microbiological contamination, stated in the EU Regulation 2073/2005 (EC, 2005). However, in the aforementioned regulation, edible insects are not considered. They could be equated to traditional meat or fish, but since their particular nature, specific microbiological criteria should be formulated. In order to overcome this problem, some European countries, such as Belgium and the Netherlands, where insects consumption is already allowed, have fixed microbiological limits for edible insect (FASFC, 2014a,b; Bureau Risicobeoordeling en Onderzoeks-programmering, 2014).

In order to guarantee high safety standard for the final consumer, suitable processing pathways should be integrated in the insect value chain. In this context, the

present review aims to illustrate the current scientific knowledge in edible insect processing, focusing on the traditional pathways and providing an overview on possible future processing routes.

2. Pre-processing

Pre-processing technologies represents the first step of each edible insects processing route and mainly consist of insect harvesting/separation from the substrate residuals, insect inactivation/killing, removal of wings/legs, and washing (Rumpold and Schlüter, 2013).

Once the insects have grown to the desired size or reach a certain age, they are harvested either manually or automatically. Larvae such as mealworms and lesser mealworms are usually separated from their substrate by sieving, while crickets and grasshoppers, which typically live apart from the feeding source, can be harvested by picking them from their rearing cage or by shaking them out of the crevices they hide in. Some farms apply a starvation period (Figure 1) before harvesting by separating the insects from their food (Garofalo *et al.*, 2019). This causes the insects to empty their intestines (Finke, 2002), which, according to the breeders, results in better taste and cleaner products with reduced microbial load (Fernandez-Cassi *et al.*, 2019). However, Wynants *et al.* (2017) reported that starvation of yellow mealworms did not reduce the total viable counts (TVC) or change the bacterial community composition consistently.

Following harvesting, the insects undergo several post-harvest treatments before being consumed. If not sold as living species, killing the insects is the first step. Freezing, drowning in hot or boiling water and steaming are some of the most common methods used for this purpose. Killing is a key step in insect processing since it can affect various final product quality parameters including microbial load, proximate composition, colour and taste (Adámková *et al.*, 2017; Farina, 2017; Larouche *et al.*, 2019). Larouche *et al.* (2019) compared blanching, desiccation, freezing, high hydrostatic pressure, grinding and asphyxiation (100% CO₂; 100% N₂; vacuum conditioning) as killing methods on black soldier fly larvae. They reported blanching (boiling water for 40 s) to be the most effective method in terms of time duration, final larval moisture, lipid oxidation, microbial load and colour alteration. However, due to the high TVC and presence of pathogenic microorganisms in the resulting product, a further stabilisation process was recommended (Larouche *et al.*, 2019). Variable results have been reported for effect of killing methods on nutritional composition of different insects. Adámková *et al.* (2017) observed lower fat content in mealworm killed by direct immersion in boiling water compared to freezing. However, Caligiani *et al.* (2019) and Singh *et al.* (2020) observed no difference in lipid concentration of *Hermetia illucens* and

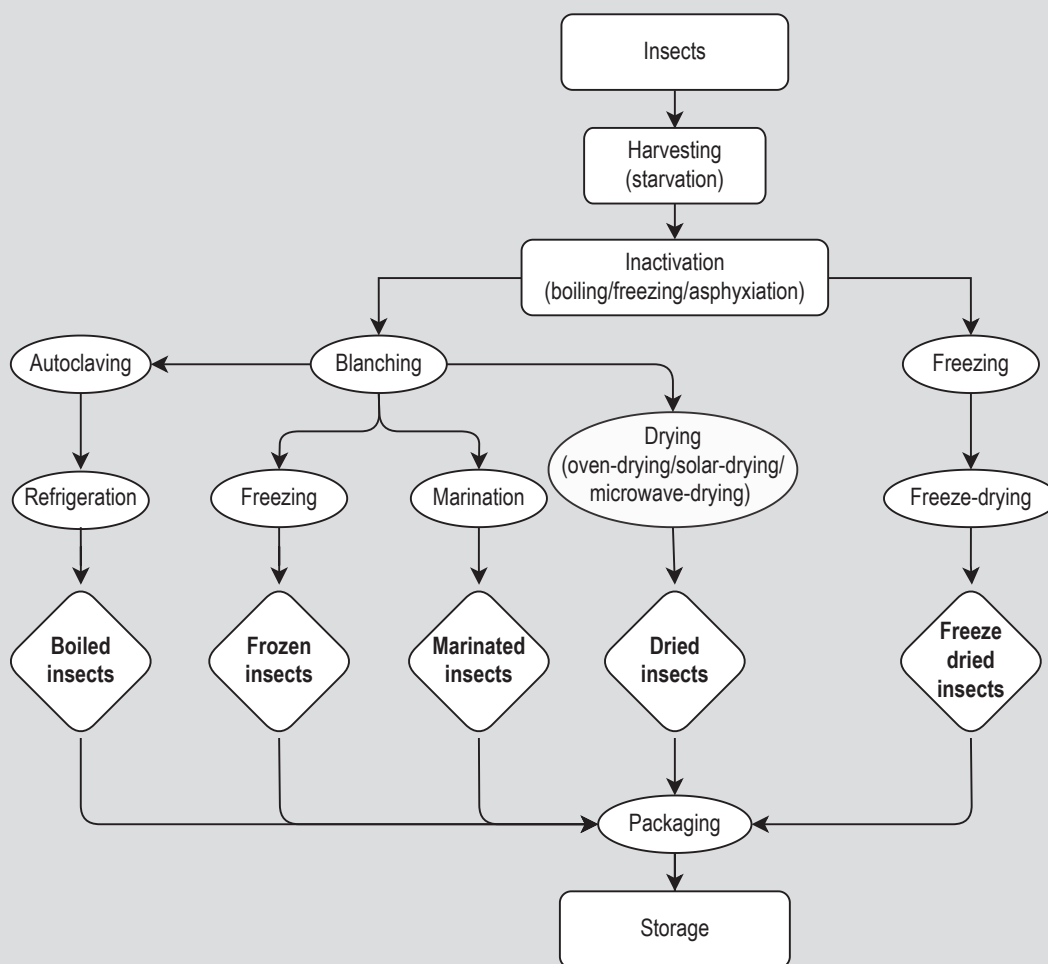


Figure 1. Processing pathways for the production of whole (boiled/frozen/oven-dried/freeze-dried/microwave-dried/marinated) edible insects.

Acheta domesticus killed by freezing (-20°C) or blanching (100°C , 40 s). Additionally, higher ether extract has been recorded when crickets were killed by asphyxiation (3 hour in plastic bag at room temperature).

The killing method can influence physiochemical properties as well. For instance, higher luminosity and lower browning have been detected in house crickets killed with CO_2 (40 min) followed by blanching (40 s), while reduction in taste and lower pH have been displayed when crickets were frozen before cooking. This was explained by the *ante-mortem* stress, which is responsible for an increasing glycolytic activity, with higher conversion of glycogen into lactic acid (Farina, 2017).

In order to reduce the microbiological load, killing process can be coupled with a rinsing/washing step, as it is already applied in traditional food products such as raw fruits and vegetables (Yoon and Lee, 2018) or carcasses and fresh meat (Huffman, 2002). However, experiments conducted by Wynants *et al.* (2016, 2017) and Ng'ang'a *et al.* (2019)

on mealworm larvae and wild harvested *Ruspolia differens* showed no reduction in TVC and in mesophilic aerobic count, Enterobacteriaceae, bacterial endospores, yeast and moulds when 1 min of washing treatment with tap water or sterile water were applied (Ng'ang'a *et al.*, 2019; Wynants *et al.*, 2016, 2017). Better results have been obtained in lesser mealworms with sterile distilled water containing antimicrobial substances such as ethanol, hydrogen peroxide or sodium hypochlorite (Crippen and Sheffield, 2006). Although these concepts can be extended to dead insects, the washing experiments aforementioned were applied on living larvae. Use of washing procedures on dead insects has been described by Fröhling *et al.* (2020). In this study, three successive washing steps with tap water were applied on frozen/thawed crickets. Although TVC of crickets was not affected by washing procedure, a reduction of approximately one logarithmic cycle was detected for total aerobic mesophilic viable count, Enterobacteriaceae, *Escherichia coli*, *Bacillus cereus*, *Clostridium perfringens*, *Enterococcus* spp., *Staphylococcus* spp., yeasts and moulds (Fröhling *et al.*, 2020).

3. Conventional processing routes

Although pre-processing represents the first step to connect from rearing to consumption, it cannot be considered decisive for ensuring food safety. Further processing techniques are required to obtain a product suitable for consumption. In the following section, the common conventional processing routes applied in the edible insects sector are described. Current scientific results as well as positive aspects and challenges observed for each step are discussed.

Processing of whole insects

Whole edible insects are usually marketed in chilled ($T \leq 5^\circ\text{C}$), frozen ($T \leq -18^\circ\text{C}$) or dried form. Dried insects have a low moisture and water activity, therefore can be stored stable for a longer time (up to months) at room temperature (Kamau *et al.*, 2018). When cold and freezing storage are used, the cold chain should be continuously maintained during the storage (James, 1996). Even though some reduction in TVC has been detected after freezing (Grabowski and Klein, 2017a; Ssepuuya *et al.*, 2016), both cold storage and freezing cannot be considered as decontamination steps as they can only decelerate or delay microbiological growth and chemical deterioration (Kamau *et al.*, 2020). A certain microbial inactivation can be reached with drying processes, despite several studies displayed high residual microbial count on dried insects (Vandeweyer *et al.*, 2017a, 2018; Wynants *et al.*, 2018). Therefore, in order to guarantee high food safety standards and extend the shelf-life, raw edible insects should be subjected to a series of steps to ensure overall safety. An overview of possible processing pathways for production of whole edible insects is presented in Figure 1.

Every processing route should be organised in 3 steps: (1) harvesting and pre-processing; (2) decontamination; and (3) packaging and storage. Several decontamination methods have been proposed on edible insects. These comprise blanching, cooking/boiling, steaming, marination, drying, smoking, toasting and their combinations. Each operation will have a different impact on the chemical and microbiological properties of the final product. For example, Nyangena *et al.* (2020) compared the effect of toasting (5 min, 150°C), boiling (5 min, 96°C), solar-drying (2 days, $50\text{--}60^\circ\text{C}$, 15–25% RH) and oven drying (2 days, 60°C) on *A. domesticus*, *H. illucens*, *Spodoptera littoralis* and *R. differensis*. They observed that in all the species, an important reduction could be detected for TVC and *Staphylococcus aureus* (5–6 log cycles), yeasts-moulds and *Salmonella* spp. (lower than detection limits) when toasting or boiling were used. On the contrary, solar-drying and oven-drying could not guarantee the same results, if not preceded by toasting or boiling (Nyangena *et al.*, 2020). The presence of pathogenic microorganisms on edible

insects after drying process represent an important safety concern, whereby dried insects should be reheated before consumption (Grabowski and Klein, 2017b). However, drying at 100°C for 4 hours was able to reduce about 5–8 log cycles of the microbial counts (TVC, total aerobic, *E. coli*, *Enterobacteriaceae*, *Enterococcus* spp., *B. cereus*, *C. perfringens*, *Staphylococcus* spp., yeasts and moulds) in *A. domesticus* (Fröhling *et al.*, 2020). Similar reduction levels have been obtained for TVC (from 7.5 to <1.7 log cfu/g), *Enterobacteriaceae* (from 6.8 to <1 log cfu/g) and bacterial endospores (from 2.1 to <1 log cfu/g) on *Tenebrio molitor*, *Brachytrupus* spp and *A. domesticus* after either boiling in water or roasting for 10 minutes (Klunder *et al.*, 2012).

A very common process in the food industry is blanching. Blanching allows the inactivation of vegetative microorganisms (but not bacterial spores). It refers to a process where a food is placed inside boiling water for short time (from few seconds to more than 10 minutes, depending by the product) and then cooled in cold water (Xiao *et al.*, 2014). It has been tested on edible insects by Vandeweyer *et al.* (2017b), who compared the effect of blanching for 10, 20 or 40 seconds, with or without microwave drying (16 microwave sources of 2 kW) on *T. molitor* larvae. They observed a significant reduction (from 8 log cfu/g to 1.3 log cfu/g) in total aerobic count when blanching for 40 seconds was followed by microwave treatment for at least 16 minutes (residual moisture 0.6%). Lower microbial reductions were detected when blanching was followed by shorter microwave treatments or no microwave process was applied. Blanching was able to reduce the number of *Enterobacteriaceae* and fungi (yeasts and moulds) below the detection limits (1 log cfu/g for *Enterobacteriaceae* and 2 log cfu/g for fungi), but an increase in bacterial endospores was observed (Vandeweyer *et al.*, 2017b). Similar results have been obtained by Vandeweyer *et al.* (2018) who studied the evolution in microbial counts during processing and storage of *Gryllus sigillatus*. They observed an initial reduction in TVC after smoking (80°C , 40 min, residual moisture 5.1%) or smoking and oven-drying (80°C , overnight, residual moisture 2.2%). However, an increase in TVC and endospores was displayed during the storage at room temperature (Vandeweyer *et al.*, 2018). Therefore, in order to obtain a final product safe for the consumer, alternative storage methods should be tested.

Still under-explored techniques in edible insects processing and storage are marination (treatment of raw meat with several ingredients to improve sensorial parameters and improve microbiological safety; Williams, 2012) and fermentation (subjecting food to the activity of microorganisms or enzymes, in order to obtain desirable sensorial and nutritional characteristics and extend the shelf life (Campbell-Platt, 1987)). While fermentation is usually conducted on insect powder or paste, marination can be

realised on whole insects. In a study from Borremans *et al.* (2018), effect of marination technology was analysed on blanched (40 s) *T. molitor* larvae. The authors concluded that 6 days of marination in red wine or soy sauce could inhibit the microbial growth, allowing shelf life extension of at least 7 days. However, marination might be responsible for germination of bacterial endospores, which might represent an issue for longer storage periods (Borremans *et al.*, 2018).

Choice of the most suitable processing methods is a key and should involve the consideration of the initial raw material and the final product that would be obtained. Besides microbial safety, choice of processing technique should also consider nutritional and sensorial parameters. For example, blanching has shown to be responsible for luminosity value and non-enzymatic browning (Azzollini *et al.*, 2016) although it is effective inactivating browning enzymes (Tonnejck-Srpova *et al.*, 2019). Another common effect connected with processing is the increase in protein solubility (Womeni *et al.*, 2012). However, these effects cannot be generalised for all insect species. For example, decrease in protein digestibility was observed on *R. differens* subjected to toasting (150 °C, 5 min) and solar drying (30 °C, 40% RH), while the same process applied on *Macrotermes subhyllanus* has not shown the same results (Kinyuru *et al.*, 2010). Processing could also be responsible for nutrient loss. It has been shown for *Hemijana variegata* subjected to sun drying (Egan *et al.*, 2014), while reduction in vitamin B12 has been detected in mealworm exposed to microwave drying (14 sources of 2 kW for 24 min) (Lenaerts *et al.*, 2018). Whereas, no proximate composition alteration was observed when oven drying (60 °C, 24 h) was used on *R. differens* (Fombong *et al.*, 2017). Reduction in protein and *in vitro* true dry matter digestibility have been detected in *Imbrasia belina* (Madibela *et al.*, 2007), *Eulepida mashona* and *Henicus whellani* (Manditsera *et al.*, 2019) after 1 hour of boiling process. For the last two species, boiling without roasting operation, was also responsible for reduction in zinc and iron bio-accessibility (Manditsera *et al.*, 2019). Conversely, 5-7 minutes of roasting could improve the calcium availability in mopane caterpillar (Madibela *et al.*, 2007).

Insect meal production

Sometimes edible insects require to be formulated into homogenous forms, such as powder or paste (Figure 2). In such cases, milling or grinding processes are required. Edible insects can be milled when still fresh or after blanching (wet milling) or after a drying process (dry milling). Wet milling is usually applied at industrial level because it results into fluid material, whereby it will be easy to handle along the production line (Dossey *et al.*, 2016). However, due to the high moisture content, the resulting material is microbially unstable (De Smet *et al.*, 2019). It

should be kept at low temperature (≤ 5 °C) and used within a short time (De Smet *et al.*, 2019). Conversely, dry milling results in a dry powder, which shows extended shelf-life and can easily be marketed and stored at room temperature (Klunder *et al.*, 2012).

In order to extend the shelf life and have a stable product, drying operations can also be performed after the wet milling process for obtaining a powder (Dossey, 2015). When insect paste has to be dried, spray drying is usually preferred. It is a drying method where the paste is propelled into hot air. In this way, every single drop of the fluid is dried singularly; therefore, the drying process happens in a short time and results in homogeneous products and with better nutritional properties (Bhandari *et al.*, 2008; Dossey *et al.*, 2016; Son *et al.*, 2019). However, since spray-drying cannot be used for products with a high fat level, drum drying is often adopted (Dossey *et al.*, 2016). Here, the insect paste is in contact with a warm surface until it is dry (Tang *et al.*, 2003). The result is a faster drying of the layer in direct contact with the drier surface and low drying of the remaining bulk. Therefore, the final powder can be inhomogeneous and characterised by low quality (Courtois, 2013; Dossey *et al.*, 2016). Table 1 shows an overview of studies where positive and negative effects connected to traditional drying technologies have been evaluated on edible insects.

Possible processing pathways resulting in several types of insect meal are shown in Figure 2 (whole fat insect meal) and 3 (defatted insect meal). Defatted insect meal is produced by extracting fat from the insect. This operation can be performed with chemical (Ravi *et al.*, 2019; Son *et al.*, 2019), physical (Arsiwalla and Aarts, 2015) or mechanical (Azagoh *et al.*, 2016; Tschirner and Simon, 2015) treatments, and results in final products with different physiochemical and sensorial characteristics (Son *et al.*, 2019) and longer shelf-life (Nadarajah *et al.*, 2015; Temba *et al.*, 2017). Defatting operation can be carried out before (Figure 3, pathways B and C) or after (Figure 3, pathway A) the milling. If milling foregoes the defatting process, enzymatic hydrolysis might occur. It is due to the action of lipases and proteases present inside the insect cells and released into the meal when the insects are pulverised. Further enzymatic hydrolysis can be reached by adding commercial enzymes to the insect paste, as suggested by Arsiwalla and Aarts (2015). In order to promote the physical separation and/or the enzymatic hydrolysis, water should be present; therefore, a drying process is conducted on the defatted paste (Figure 3, pathway A). When defatted insect meal is produced by a mechanical defatting process (Figure 3, pathway B and C), milling is usually conducted after oil extraction (Azagoh *et al.*, 2016; Son *et al.*, 2019), while drying can be performed on the final defatted paste (Figure 3, pathway B) or on the whole insect (Figure 3, pathway C). Finally, solvent defatting operation is usually performed on dry material

Table 1. Summary of studies exploring the effect of different drying processes on edible insects.

Insect species	Drying methods	Notable effect	Reference
<i>Acheta domesticus</i> , <i>Hermetia illucens</i> , <i>Spodoptera littoralis</i> , <i>Ruspolia differens</i>	Oven drying Freeze drying Solar drying	Oven drying decreased bacterial populations, yeasts and mould. Solar drying had no effect. All three drying processes combined with boiling eliminated bacterial populations, yeasts, moulds.	Nyangena <i>et al.</i> (2020)
<i>Rhynchophorus phoenicis</i>	Solar drying Oven drying Smoke drying	Smoke drying was suggested as the preferable method as it maintains the lipid profile, while solar drying increased peroxide value.	Tiencheu <i>et al.</i> (2013)
<i>Sternocera orissa</i>	Oven drying Freeze drying	Oven drying method led to a higher composition of the material in compare to freeze drying	Shadung <i>et al.</i> (2012)
<i>Imbrasia epimethea</i>	Oven drying Solar drying	The monosaturated fatty acid content was reduced.	Lautenschläger <i>et al.</i> (2017)
<i>Polyrhachis vicina</i>	Solar drying	Aldehydes, increase of free fatty acid content, decrease of ketone content and hydrocarbons were observed.	Li <i>et al.</i> (2009)
<i>R. differens</i>	Oven drying Freeze drying	Both processes led to no significant differences in composition and product quality.	Fombong <i>et al.</i> (2017)
<i>Tenebrio molitor</i>	Oven drying Microwave-assisted drying	Microwave-assisted drying, combined with 40 s of blanching for less than 16 min did not reduce water activity lower than 0.6.	Vandeweyer <i>et al.</i> (2017b)
<i>T. molitor</i>	Oven drying Freeze drying Fluidised bed drying	Higher temperatures of drying caused darkening and shrinking due to browning reactions and tissue disruption.	Purschke <i>et al.</i> (2018a)
<i>T. molitor</i>	Fluidised bed drying Microwave-assisted drying Freeze drying Vacuum drying Hot air drying	Freeze drying oxidised the lipid fraction. Protein solubility was higher with vacuum drying and freeze drying and decreased with microwave-assisted drying.	Kroncke <i>et al.</i> (2018)
<i>H. illucens</i>	Oven drying Microwave-assisted drying	Protein digestibility was higher with oven drying, since microwave treatment polymerised protein molecules.	Huang <i>et al.</i> (2019)
<i>Musca domestica</i> L.	Oven drying Solar drying	Oven dried samples contained more proteins and less fat	Aniebo and Owen (2010)
<i>Macrotermes subhyllanus</i> , <i>R. differens</i>	Solar drying	Solar drying of termites and grasshoppers decreased vitamin content in both species and protein digestibility of the grasshoppers.	Kinyuru <i>et al.</i> (2010)
<i>Locusta migratoria manilensis</i> , <i>Bombyx mori</i>	Freeze drying Oven drying Microwave-assisted drying	Oven and microwave drying changed the sensory characteristics of the materials in a way that they were less acceptable.	Mishyna <i>et al.</i> (2020)

(Ravi *et al.*, 2019; Son *et al.*, 2019), which should be milled to ultrafine powder in order to increase the surface and to improve the solvent penetration (Son *et al.*, 2019). However, although defatting yield with solvent is higher than with other methods (Son *et al.*, 2019), solvent defatting operations are not preferred for both, environmental and sensorial aspects. Indeed, n-hexane defatted mealworm meal showed less flavour, lower colour preservation and consumer acceptability than pressure-defatted powder (Son *et al.*, 2019).

The type of blender used for meal production can also affect the final properties of the insect powder/paste. For example, Son *et al.* (2019) observed that mealworm defatted meal obtained by using a jet-mill showed lower humidity, higher luminosity, higher particle size uniformity and higher consumer acceptance than powder produced by pin, hammer or cutter mill (Son *et al.*, 2019).

Apart from the three pathways already described, several other processing routes have been described for insect meal production (Borremans *et al.*, 2020a,b; Bußler *et al.*,

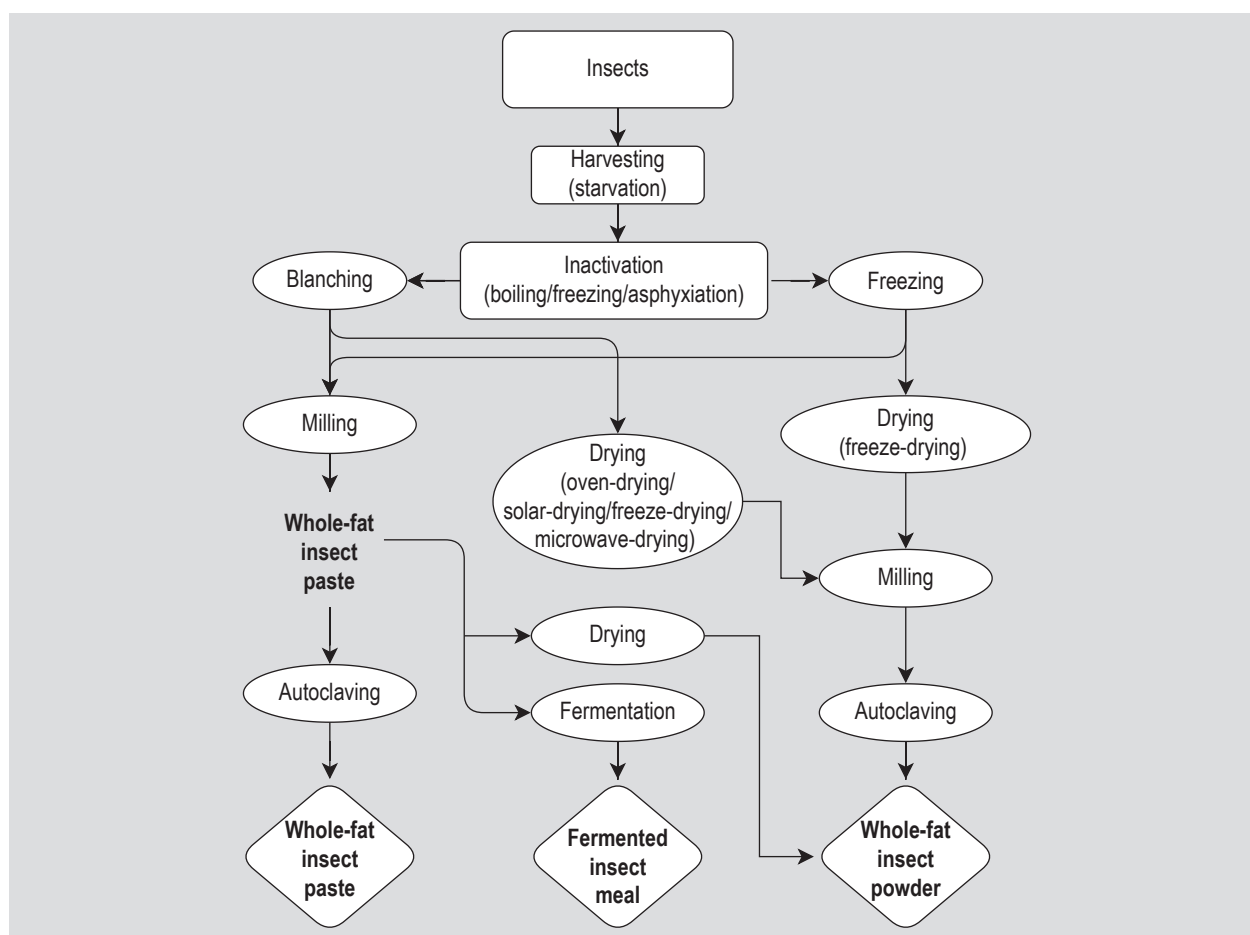


Figure 2. Processing pathways for the production of whole-fat and fermented insect meal.

2016b; Fröhling *et al.*, 2020; Purschke *et al.*, 2018a,b,c; Vandeweyer *et al.*, 2018). Depending on the process goal and the insect species, the order of several operations may vary (Stoops *et al.*, 2017).

The most critical step along each processing pathway is the thermal treatment, which is important to assure food safety. Microbial decontamination can be performed on whole insects (before milling operation) (Kamau *et al.*, 2020) or directly on the powder/paste resulting from the milling process (Bußler *et al.*, 2016a,b) (Figure 2). Applying the decontamination process on meal is preferred, since milling operations break down the insect body and a release of the insect gut microbiota is obtained increasing the total microbial count (Fröhling *et al.*, 2020; Klunder *et al.*, 2012; Rumpold *et al.*, 2014). Indeed, microorganisms at the surface of food materials will be more susceptible to high temperature (Rumpold *et al.*, 2014); therefore, acceptable results can be reached with lower temperature or shorter treatment time. In this way, less impact on physio-chemical and sensorial characteristics of the final product is realised (Fröhling *et al.*, 2020). However, every time high temperatures are applied on insect meal, it may result in burnings of the powder/paste (Arsiwalla and

Aarts, 2015). Concerning the physicochemical stability, the presence of browning and lipolytic enzymes should be considered. They can be responsible for browning, lipid oxidation and rancidity of insect paste. Therefore, in order to prevent undesirable reactions, a blanching process can be realised on the whole insects (Figure 3, pathways B and C) as suggested by Azagoh *et al.* (2016). Nevertheless, when blanching is performed, the adding of water can result in loss of soluble nutrients and higher energy is required for drying (Fröhling *et al.*, 2020). However, blanching of whole insect can be responsible also for microbial inactivation, resulting in low number of *Enterobacteriaceae* (Stoops *et al.*, 2017), but possible reactivation of bacterial spores should also be considered (Stoops *et al.*, 2016). For this reason, further decontamination treatment should be carried out in the next steps. Besides drying and heat based operations, an interesting stabilisation method applied on insect-based food is fermentation (Figure 2). The ability of this technology to ensure quality and safety of insect-based food has been investigated in several studies. For example, Patrignani *et al.* (2020) used *Yarrowia lipolytica* and *Debaryomyces hansenii* strains to produce cricket flour hydrolysates with high food safety, functionality, sensory and technological properties. The hydrolysates obtained

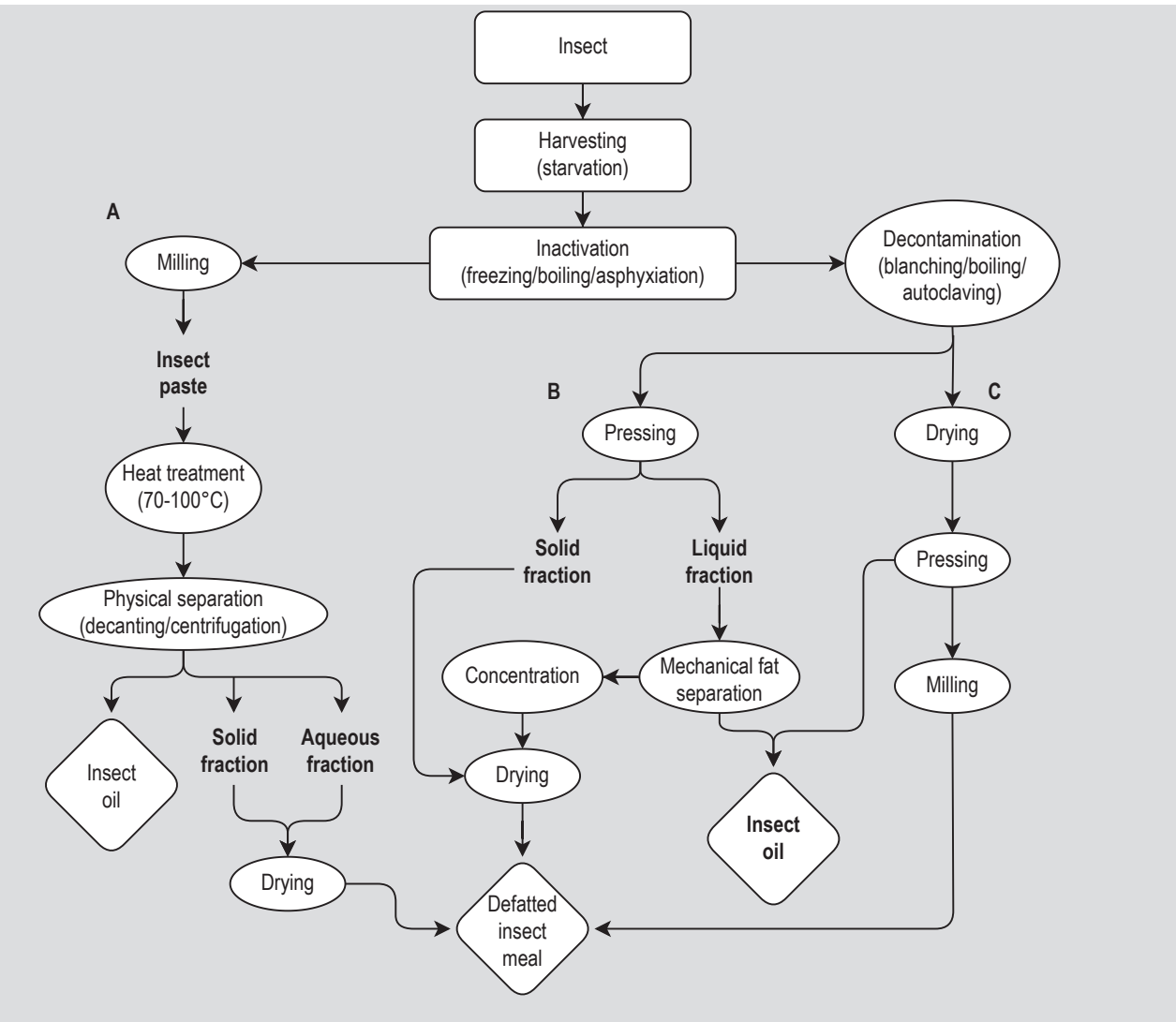


Figure 3. Processing pathways for production of defatted edible insect meal and insect oil: (A) as described by Arsiwalla and Aarts (2015), (B) analogous to fish meal production, (C) dry processing pathway.

by fermentation were characterised by a reduced chitin content and higher contents of antimicrobial substances (acetic acid, short chain fatty acids, chitosan, and GABA) and health-promoting molecules (arachidonic and linolenic acids, GABA, AABA, and BABA) when compared to the control. Borremans *et al.* (2018) observed that rapid acidification during fermentation, able to prevent microbial growth, could be reached within three days when blanched (40 s) paste of *T. molitor* was inoculated with a commercial starter containing *Pediococcus acidilactici*, *Lactobacillus curvatus* and *Staphylococcus xylosus* (Borremans *et al.*, 2018). However, the authors detected some lipid oxidation during refrigerated storage (4 °C) of the fermented paste. In order to prevent the oxidation, several additives have been tested. Borremans *et al.* (2020b) observed that sodium nitrite (150 mg/kg) and sodium lactate (60% w/w solution, 50 g/kg) are both able to reduce lipid oxidation. Different results were obtained by De Smet *et al.* (2019)

who observed higher lipid oxidation of fermented insect paste stored for three weeks at 4 °C, when sodium nitrite or sodium lactate, rather than no additives, were used. Use of additive has displayed also a reduction in sulphite reducing bacteria in the fermenting paste (De Smet *et al.*, 2019). It is an important aspect because these bacteria are responsible for reduction of sulphite to hydrogen sulphite, compound associated with the unpleasant ‘rotten egg’ odour (Borremans *et al.*, 2018).

Storage of insect-based food

Although the aforementioned techniques can guarantee microbial safety, they cannot prevent the re-contamination of the product in the subsequent working steps. In order to guarantee microbiological safety for the consumer, appropriate storage methods with respect to the specific product properties should be applied. Refrigeration and

freezing, as well as air containing packaging, vacuum packaging and modified atmosphere packaging (MAP) are common preservation methods used for edible insect products. Klunder *et al.* (2012) observed that crickets boiled for 1 minutes could maintain low TVC (4.1 log cfu/g) over 16 days when stored at 5–7 °C, while TVC higher than 10 log cfu/g was reached after 2 days in room temperature storage (28–30 °C) (Klunder *et al.*, 2012). Similar results have been obtained on blanched *T. molitor* larvae stored at 3 °C for 6 days (Vandeweyer *et al.*, 2017b), while stable TVC over 180 days has been found on refrigerated (about 5 °C) black soldier fly larvae (Kamau *et al.*, 2020). Refrigeration has also shown promising results for storage of fermented or marinated insects, guaranteeing microbiological and chemical stability for up to two and four weeks respectively (Borremans *et al.*, 2018, 2020b; De Smet *et al.*, 2019). Cold storage can provide good lipid stability and thus the sensorial quality of insect products (Kamau *et al.*, 2020; Lee *et al.*, 2020; Tiencheu *et al.*, 2013). However, since refrigeration temperatures are not able to inactivate lipases, enzymes responsible for lipid oxidation, freezing can be applied to inhibit enzyme activities (Tiencheu *et al.*, 2013).

Important differences in terms of lipid oxidation and microbial growth were also observed when different packaging was used: insect meal packaged in polypropylene bags and stored at room temperature showed higher lipid oxidation than insect meal packaged in polyethylene bags (Kamau *et al.*, 2020). It was attributed to the permeability of polypropylene to water, which was considered responsible for propagation of moulds able to produce lipases (Kamau *et al.*, 2020). Combined effects of packaging and storage conditions have been tested also by Ssepuuya *et al.* (2016, 2019) who observed that storage at room temperature (20 °C) could guarantee microbiological, chemical and sensorial stabilities only if opaque vacuum packaging was applied (Ssepuuya *et al.*, 2016, 2019). An alternative to vacuum packaging is MAP. It has been investigated by Stoops *et al.* (2017), who observed that MAP (60% CO₂ 40% N₂) reduced microbial growth guaranteeing at least 21 days of microbiological stability for insect paste stored at 4 °C (Stoops *et al.*, 2017). However, they did not observe any reduction in bacterial spore counts.

Use of MAP may also be helpful to reduce the moulds contamination of dried whole insects or insect powder. At the best of our knowledge, no study has been conducted in this area, but this technology can be extended to insects from other dried food (Rodriguez *et al.*, 2000). MAP can represent an important step in edible insect shelf-life extension because moulds are responsible for mycotoxin production, which can be a fundamental safety issue in edible insects when the water activity is higher than 0.6 (Kamau *et al.*, 2018). In a study conducted by Kachapulula *et al.* (2018), high moulds proliferation has been observed on dried caterpillars and termites after 7 days of storage at

room temperature (31 °C). It was accompanied with high aflatoxin contamination, which made the insects not safe for human or animal consumption (Kachapulula *et al.*, 2018).

Extraction of valuable compounds from insects

Despite a large number of studies aiming to use whole insects or insect meal as food or food ingredient, many consumers, especially from the western world, may still not accept them (Chen *et al.*, 2009). In order to overcome these limitations and promote their consumption, isolation of protein and fat-rich fractions can be performed. The resulting products may then be used as ingredients for both industrial processing and culinary home-made preparation.

Isolation of protein has been performed from insect meal obtained from several species such as migratory locust (Clarkson *et al.*, 2018; Purschke *et al.*, 2018c), desert locust (Mishyna *et al.*, 2019) house cricket (Laroche *et al.*, 2019; Ndiritu *et al.*, 2017), mealworm (Bußler *et al.*, 2016b; Kim *et al.*, 2019; Laroche *et al.*, 2019; Yi *et al.*, 2013, 2017; Zhao *et al.*, 2016) black soldier fly (Bußler *et al.*, 2016b; Caligiani *et al.*, 2018; Soetemans *et al.*, 2019). Extraction conditions, as well as solvent and insect species can strongly influence extraction yield and properties of the isolated proteins. Satisfactory protein yields have been obtained by using alkaline solutions (Laroche *et al.*, 2019; Mintah *et al.*, 2020; Mishyna *et al.*, 2019; Zhao *et al.*, 2016) since pH values between 10 and 12 have displayed to lead to higher insect protein solubility (Bußler *et al.*, 2016b; Purschke *et al.*, 2018b,c; Udamsil *et al.*, 2019; Yi *et al.*, 2017; Zhao *et al.*, 2016). However, use of chemical solvents has shown negative effects on protein functionality, impacting several parameters such as emulsion capacity, foaming capacity and foaming stability (Ndiritu *et al.*, 2017). In order to prevent it, aqueous extraction may be performed. However, lower extraction yields have been shown when aqueous solutions of ascorbic acid have been used (Amarender *et al.*, 2020; Ndiritu *et al.*, 2017; Yi *et al.*, 2013). Improved results have been reached by using NaCl, as displayed on *T. molitor* (Yi *et al.*, 2017), or increasing pH (Purschke *et al.*, 2018b), temperature, solid/liquid ratio and/or extraction time (Bußler *et al.*, 2016b; Mintah *et al.*, 2020). Defatting can also increase the protein extract yields, as shown for several insect species (Amarender *et al.*, 2020; Kim *et al.*, 2019).

Several extraction systems have been implemented for recovery of fat from edible insects. Common fat extraction methods comprises Folch, Soxhlet, Supercritical CO₂ and aqueous extraction (Laroche *et al.*, 2019; Ramos-Bueno *et al.*, 2016; Ravi *et al.*, 2019; Tzompa-Sosa *et al.*, 2014). Choice of the method should consider the insect species because the same method applied on several species can results in different yields, as shown by Laroche *et al.* (2019), Pan *et al.* (2012), Ramos-Bueno *et al.* (2016) and Tzompa-Sosa *et al.* (2019, 2014). Moreover, different extraction systems, as

well as different solvents, lead to various extraction yield when applied on the same insect species (Amarender *et al.*, 2020; Feng *et al.*, 2020; Laroche *et al.*, 2019; Mariod *et al.*, 2010). Fatty acid profiles of the extract can change with the extraction system as well (Laroche *et al.*, 2019; Purschke *et al.*, 2017; Ramos-Bueno *et al.*, 2016; Ravi *et al.*, 2019; Tzompa-Sosa *et al.*, 2014).

An interesting bioactive compound that can be extracted from insects is chitin. It is the precursor of chitosan, an amino-polysaccharide characterised by important biological activities (Mohan *et al.*, 2020). The process for chitin extraction consists in four sequential steps: delipidation, deproteinisation, demineralisation, decolorisation (Mohan *et al.*, 2020). The aim of this processing pathway is to remove all the extractable compounds from the insect powder in order to obtain the insoluble chitin fraction. This method has been implemented on a wide range of insect species, such as *Bombyx mori* (Luo *et al.*, 2019; Simionato *et al.*, 2014), *T. molitor* (Luo *et al.*, 2019; Song *et al.*, 2018), *Zophobas morio* (Shin *et al.*, 2019; Soon *et al.*, 2018), *H. illucens* (Caligiani *et al.*, 2018; Purkayastha and Sarkar, 2020), *Musca domestica* (Jing *et al.*, 2007; Kim *et al.*, 2016), *Apis mellifera* (Marei *et al.*, 2016, 2019), *Gryllus bimaculatus* (Kim *et al.*, 2017), *Schistocerca gregaria* (Marei *et al.*, 2016) and others. In order to reduce the use of chemical compounds, new eco-friendly chitin extraction systems have been developed. For example, the aforementioned method has been enhanced by using a sustainable deep eutectic solvent, which has shown attractive results on chitin extraction from black soldier fly (Zhou *et al.*, 2019). Furthermore, biological methods, based on fermentation with bacterial strains isolated from mealworm surface, have shown to be strong candidates for replacement of chemical demineralisation (Da Silva *et al.*, 2017).

4. Emerging technologies in insect processing

Recent advances in agri-food sector have shown that the emerging technologies (e.g. high hydrostatic pressures (HHP), pulsed electric fields (PEF), ultrasound (US) and CAPP) are promising alternatives for sustainable eco-friendly processing with negligible environmental impacts (Pojić *et al.*, 2018). These technologies aim to improve the safety and the quality of final products and enhance processing efficiency. However, requirement of highly trained operators and presence of remarkable initial investments may represent important hurdles for their spreading (Priyadarshini *et al.*, 2019). Legislative aspects need to be considered as well. For example, in Europe the novel food regulation (EU Regulation 2283/2015) considers products obtained by using emerging technologies as novel food if the applied technology was not used within the EU before 15 May 1997 and shows an impact on nutritional composition, structure or safety of the obtained food, which cannot be compared with the same food produced using

traditional technologies. Since HHP, PEF and US are already applied in industrial food processing, the relation between emerging technologies and novel food legislation is not always clear and should be evaluated case by case every time one of these technologies is used.

In the present section, the most applied innovative technologies are described. A general overview of each technology, its fundamental effects and main applications in the food industry are given. Afterwards, possible applications of emerging technologies in the edible insects sector are discussed.

Emerging technologies

High hydrostatic pressure is a discontinuous or semi-continuous process that involves a fluid, submitted at a pressure in a range of 100-1000 MPa (Koutchma, 2014), surrounding the product to be treated at 0 to 120 °C (Aganovic *et al.*, 2021). However, pressure in the range of 200 up to 600 MPa at ambient or chilled temperature is usually applied in commercial food applications, with holding times rarely longer than 5 min (Aganovic *et al.*, 2021). The pressure can damage the microbial cells, leading to the inactivation of microorganisms at significantly lower temperature than conventional thermal processing and resulting in a better final product quality (Kashiri *et al.*, 2018). In food processing applications, HHP alone or in combination with heat can cause various physical, chemical, or biological changes in food, responsible for formulation of modified foods and ingredients (Bolat *et al.*, 2021; Hugas *et al.*, 2002; Rumpold *et al.*, 2014; Ugur *et al.*, 2020). Further, HHP can also be used to support the nutrient extraction from food (Preece *et al.*, 2017), to maximise the extraction yield of oils (Andreou *et al.*, 2020) and to improve product acceptability through the degradation of allergenic compounds which are naturally present in some food products (Boukil *et al.*, 2020; Li *et al.*, 2012; Penas *et al.*, 2011). However, HHP may have a negative impact on protein stability, resulting in loss of important nutritional components and changing the food texture (Boukil *et al.*, 2020; Tonnejck-Srpova *et al.*, 2019; Wang *et al.*, 2016). Moreover, when food is treated with HHP at moderate temperature, residual contamination with bacterial spores can still be detected (Campbell *et al.*, 2020; Kashiri *et al.*, 2018; Stoica *et al.*, 2013; Wang *et al.*, 2016). Therefore, the requirement of further heat operations and the high cost of HHP generating devices (Wang *et al.*, 2016), represent important challenges for using this technology for edible insect processing in food industry.

Pulsed electric field application is a relatively recent and widely employed technology in the food sector for several purposes. PEF consists of processing the food product with short high voltage electric pulses (Nowosad *et al.*, 2021). Depending on the purpose, several pulse frequencies

and exposition times can be applied. The mechanism of the technology is related to triggering a change in the cell membrane integrity with consequent enhancement of its permeability (Nowosad *et al.*, 2021). This phenomenon can be exploited in several ways, including improvement of extraction efficiency of nutritional and bioactive compounds from raw materials (Alles *et al.*, 2020; Andreou *et al.*, 2020; Kaferbock *et al.*, 2020; Rahaman *et al.*, 2020) and food waste (Franco *et al.*, 2020). Further, PEF coupled with some conventional processes, like freezing, drying or mechanical extraction of juices or oils, can lead to increase in yields, efficiency optimisation (Alles *et al.*, 2020; Andreou *et al.*, 2019; Shorstkii *et al.*, in press), and milder stabilisation treatments which allow obtaining fresh-like products with texture, flavour, colour and odour closer at their natural state (Liu *et al.*, 2020; Tao *et al.*, 2019). Hurdles for PEF application in food industry include the high initial costs (Stoica *et al.*, 2013) and its lack of activity on bacterial endospores inactivation, which requires a further treatment (Reineke *et al.*, 2015; Siemer *et al.*, 2014). However, unlike HHP, no negative impact on bioactive compounds has been detected with PEF treatment (Nowosad *et al.*, 2021; Shorstkii *et al.*, in press).

Ultrasound technology consists of mechanical acoustic waves propagating in an elastic (solid or fluid) medium (Lempriere, 2003). These waves can generate compression and decompression on the matrix particles, producing a huge amount of energy (Gallo *et al.*, 2018). High intensity ultrasound operates at frequencies between 20 and 100 kHz and the intensity ranged between 10 and 1000 W/cm². This high intensity allows them to be disruptive by inducing cavitation inside the treated medium, with consequent alterations on physical, chemical and mechanical properties (Bhargava *et al.*, 2021). In the food industry, US is usually used to improve the efficiency of several unit operations. US can be used to enhance the extraction yield by creating cavities inside the tissues and favouring the breakdown of cell walls with a reduction of time and energy used for compounds' extraction (Choi *et al.*, 2017; Mishyna *et al.*, 2019; Ojha *et al.*, 2020; Sun *et al.*, 2018). With a similar mechanism, US can also be coupled with drying process. The micro-channels generated inside the bulk because the molecular cavitation, can favour the water elimination, reducing time and temperature of the drying processes. This can lead to a higher quality of the final product (Huang *et al.*, 2020). Besides the mass transfer, US can also be used for heat transfer. For example, US can facilitate the freezing process through improved initial nucleation (Chow *et al.*, 2005). Further, US can be also used during thawing with the goal to reduce the defrosting time in order to avoid product degradation (Li *et al.*, 2020). US coupled with heat treatments (thermosonication) or high pressure (manosonication) has found application also in sterilisation and enzyme inactivation, allowing to obtain interesting results in terms of shelf-life extension, without altering the

sensorial characteristics of the food (Cameron *et al.*, 2009; Mintah *et al.*, 2019b; O'Donnell *et al.*, 2010). However, when US is not applied properly, it can be responsible for increase of temperature, with negative effects on nutritional and organoleptic characteristics (Farkas and Mohacsi-Farkas, 2011; Mintah *et al.*, 2020). Despite these drawbacks, US is the mostly used novel technology in the food sector so far (Priyadarshini *et al.*, 2019).

CAPP is a new technology with high potential, but to the authors best knowledge not applied in food industry yet. Plasma constitutes a mixture of several active chemical species, i.e. electrons, free radicals, ions, protons, excited atoms, reactive oxygen species, reactive nitrogen species, and UV radiation (Moreau *et al.*, 2008). These reactive compounds are created by applying electric or electromagnetic fields, generated at several voltages, to a gas (Ramazzina *et al.*, 2015). CAPP indicates a specific kind of plasma, generated at low temperatures (usually between 30 and 60 °C) and atmospheric pressure (Moreau *et al.*, 2008). The lower operating temperature provides interesting opportunities for shelf-life extension of several food matrices, without altering the quality parameters of temperature-sensitive products (Bußler *et al.*, 2016a; Pan *et al.*, 2019). CAPP has also shown its potential to be active not only on the viable microbial cells but also on spores, thus allowing low-temperature sterilisation of the food product (Bußler *et al.*, 2016a; Liao *et al.*, 2018; Rumpold *et al.*, 2014). The high reactivity and instability of the compounds produced during plasma treatment can also be used for inactivation of certain enzymes due to the alteration of their protein structures (Bußler *et al.*, 2016a, 2017; Han *et al.*, 2019). With the similar mechanism, CAPP can facilitate the decontamination of foods from allergens, pesticides, mycotoxins and other toxic contaminants (Gavahian and Cullen, 2020; Gavahian and Khaneghah, 2020). Another application of CAPP in the food industry is related to the modification of some food components and their physical characteristics, with the purpose to improve their quality and increase the processing efficiency (Bußler *et al.*, 2016a; Ekezie *et al.*, 2017). However, reactive oxygen species present in CAPP have shown negative activities on lipid stability, promoting lipid oxidation, which can result in off-flavour (Varilla *et al.*, 2020). CAPP can also find application in food fortification to obtain a product with specific healthy properties (Akasapu *et al.*, 2020). Furthermore, CAPP has also been applied on some by-products to extract interesting secondary nutrients as well as phenolic compounds (Bao *et al.*, 2020). Main drawbacks for CAPP application in food industry are connected with the uncertainty of find toxic residuals in food. Despite no evidences of potentially dangerous compounds in plasma treated food have been found so far (Kromm *et al.*, unpublished data; Priyadarshini *et al.*, 2019), it is recommended that each product should be assessed individually (Schlüter *et al.*, 2013).

Innovative processing routes

Novel technologies have shown their potential for different processes in the food industry; however, their use in edible insect processing is still scarce. Traditional methods, which are well known and easy to operate, are still preferred, due to the high variability of the insect-products and the high difficulty of process optimisation. However, novel technologies can lead to improved quality and safety of insect based products, assuring a larger consumer acceptance. Several studies have been performed in this regard. Table 2 offers an overview of these studies. Further, Figure 4 and 5 present possible innovative processing pathways, for the production of whole edible insects and insect products respectively, where novel processing technologies can modify, complement, or replace the conventional processing routes.

Microbial decontamination

CAPP and HHP have been studied to reach an acceptable microbial reduction grade and guarantee safety for the final consumer. Rumpold *et al.* (2014) observed that indirect plasma treatment for 10 min is very effective for surface decontamination of mealworm larvae, allowing microbial reduction of 7 log cycles. However, the same treatment has not shown any effect on gut microbial load. On the contrary, HHP is more active on gut microbial count reduction than on the surface (Kashiri *et al.*, 2018). Therefore, combination of CAPP and HHP can be a suitable alternative to traditional heat treatments in order to reach adequate microbial reduction on whole edible insects (Figure 4, pathway A). Another possibilities is to couple HHP or CAPP with traditional heat treatments realised at lower temperature and shorter time (Rumpold *et al.*, 2014). Similar reasoning can be done for insect meal. Bußler *et al.* (2016a) investigated the impact of CAPP on the microbial load of *T. molitor* flour showing reduction of the total microbial load from 7.7 to 4.3 log cfu/g. However, reduction from 6.8 log cfu/g to below detection limit has been observed when a thermal treatment for 15 min in an oven at temperatures of 120 °C was applied (Bußler *et al.*, 2016a,b). The microbial species to be inactivated should be considered as well. For example, Campbell *et al.* (2020) observed that a HHP treatment at 400 MPa for 10 min on *H. illucens* larvae is able to reduce yeasts and moulds below the detection limits, while reductions of *Enterobacteriaceae* (from 7.65 to 3.32 log cfu/g) and lactic acid bacteria (from 6.50 to 4.73 log cfu/g) were remarkable but not satisfactory. These results have confirmed the finding of Kashiri *et al.* (2018), who observed that HHP at 400 MPa for 2.5 min was responsible for a total inactivation of yeasts and moulds, but no significant effect was observed for total aerobic mesophilic microbial count, where the reduction was only 0.35 log cycles (Kashiri *et al.*, 2018).

Enzyme inactivation

In order to promote shelf life extension of food products, undesirable chemical reactions, such as enzymatic browning, should be prevented. Interesting results in terms of enzyme inactivation have been obtained when HHP treatments were used on *T. molitor* flour (Tonnejck-Srpova *et al.*, 2019). In this study, HHP has shown a potential to prevent enzymatic browning, which was comparable with traditional blanching ($L^*90 \geq 60$ after 10–11 days for HHP and $L^*90 \approx 60$ after 3–4 days for blanching). However, HHP treatment had a negative impact on the product texture, with a high amount of serum released from the mealworm powder. On the contrary, treatment at 500 MPa was able to prevent the enzymatic browning and at the same time to reduce the amount of released serum, obtaining a product with a weaker texture, therefore more desirable by the consumer (Tonnejck-Srpova *et al.*, 2019).

Drying

Novel technologies are also known for increasing efficiency and yield of basic unit operations such as drying and freezing (Bassey, 2021; Wu, 2017) (Figure 4, pathway B). Concerning edible insects, impact of PEF pre-treatment on drying time has been evaluated by Alles *et al.* (2020). They observed that PEF at 2 kV/cm and 20 KJ/kg was able to reduce the drying time of *H. illucens* pre-pupae. However, these benefits were not observed at higher electric field strength (3 kV/cm), highlighting the importance of process optimisation (Alles *et al.*, 2020; Shorstkii *et al.*, in press).

Interesting results in terms of increase in mass transfer rate and time reduction during drying process may be obtained by applying US on insects as a pretreatment and/or in combination (Figure 4, pathway B). At the best of our knowledge, no study has investigated this potentiality on edible insects. However, basic knowledge can be transferred from other food materials (Huang *et al.*, 2020).

Extraction

Traditional extraction procedures are often time consuming, inefficient and non-selective (Otero *et al.*, 2020). Ultrasound and PEF have shown important solutions for these problems. For example, in a recent study, Otero *et al.* (2020) compared two different methods (i.e. ultrasound assisted extraction and pressurised liquid extraction) and two solvents (pure ethanol and a mixture of ethanol and water 1:1 v/v) to extract oil from *A. domesticus* adults and *T. molitor* larvae. They observed that ultrasound can increase the content of polyunsaturated fatty acids and decrease the content of saturated and monounsaturated fatty acids of the extract. Despite these positive results, every extraction method was found to increase the cholesterol level of the final extract. Besides, the effect of ultrasound is strictly related to the

Table 2. Innovative technology in insect processing.¹

Process goal	Technology and processing condition	Insect species and form	Main findings	Reference
Microbial decontamination	HHP treatment (35 L vessel, at room temperature) at 400 MPa and 600 MPa; 1.5 min and 10 min	<i>Hermetia illucens</i> larvae	HHP resulted in a lower inactivation for TVC and 600 MPa was needed to achieve similar reductions against Enterobacteria, LAB, and YM.	Campbell <i>et al.</i> (2020)
	HHP treatment (250 to 400 MPa, for 1.5 to 15 min)	<i>H. illucens</i> larvae	HHP was effective against natural contaminating YM, resulting more than 5 log cycle reductions at 400 MPa for all treatments; however, a low reduction of total microbial load was achieved.	Kashiri <i>et al.</i> (2018)
	CAPP generated at sinusoidal voltage of 8.8 kV _{PP} at a frequency of 3.0 kHz using air as working gas; treatments up to 15 min	<i>Tenebrio molitor</i> flour	Total microbial load reduced from 7.72 to 4.73 log ₁₀ cfu/g in 15 min CAPP treatment.	Buřler <i>et al.</i> (2016a)
	HHP treatment (400, 500, and 600 MPa; up to 15 min)	<i>T. molitor</i> larvae	A HHP treatment at 600 MPa for 10 min resulted in an inactivation of 3 log cycles.	Rumpold <i>et al.</i> (2014)
	Indirect plasma treatment at frequency of 2.45 GHz and a power consumption in the range of 1.2 kW	<i>T. molitor</i> larvae	Plasma treatment (10 min) resulted in up to 7 log cycles reduction.	Rumpold <i>et al.</i> (2014)
Drying	PEF at 2 and 3 kV/cm; 5, 10 and 20 kJ/kg wet basis	Live <i>H. illucens</i> larvae	PEF pre-treatment can significantly reduce (up to 30%) drying time of insect biomass.	Alles <i>et al.</i> (2020)
Enzymatic hydrolysis	HHP treatment at 380 MPa for 1 min applied before or during the enzymatic hydrolysis by pepsin or Alcalase®	<i>T. molitor</i> larvae powder	HHP applied before the enzymatic hydrolysis improved the enzymatic hydrolysis by Alcalase® (but not by pepsin) at the very beginning of the process. Partial reduction of allergenic properties was observed. No alterations on the protein profile were observed.	Boukil <i>et al.</i> (2020)
	Ultrasound pretreatment at 600 W and 40±2 kHz for 30 min. Pulse interval of 15 s on and 5 s off. Temperature of 50 °C	Defatted <i>H. illucens</i> meal	US pretreatment enhance the enzymolysis reaction, by altering the molecular structure and arrangement and improving the enzyme-substrate interactions.	Mintah <i>et al.</i> (2019a)
	Ultrasound pretreatment at 600 W and 40±2 kHz for 10-20-30 min. Pulse interval of 15 s on and 5 s off	Dried defatted <i>H. illucens</i> meal	US assisted hydrolysis leads to hydrolysates with higher antioxidant activity.	Mintah <i>et al.</i> (2019b)
	Ultrasound pretreatment at 600 W and 40±2 kHz with a pulse interval of 15 s on and 5 s off	Dried defatted <i>H. illucens</i> larvae	Ultrasound pretreatment increase the sample lightness by reducing the protein hydrolysate molecular weight. Sonic treatments alter the secondary protein structure, reducing the hydrolysate turbidity and increasing the protein dispersibility.	Mintah <i>et al.</i> (2020)
	HHP treatment (4 l vessel) at 250, 300, 400 and 500 MPa for 3 min	<i>T. molitor</i> larvae	Pressure of 400 MPa prevented the enzymatic browning.	Tonneijck-Srpova <i>et al.</i> (2019)

Table 2. Continued.

Extraction	Ultrasound assisted extraction by direct sonication at 20 kHz, amplitude of 89.9 µm in continuous pulse; 15 min	Lyophilised and grounded <i>Acheta domesticus</i> and <i>T. molitor</i>	The extraction yield was dependent on the nature insect species and the solvent of extraction. Ultrasound can be used to obtain insects extracts with improved fatty acid profile.	Otero et al. (2020)
	Ultrasonic assisted aqueous extraction by sonication at 20 kHz (several parameters combinations tested)	Lyophilised and grounded <i>Clanis bilineata</i> larvae	Ultrasound treatment allowed increasing oil extraction yield and oil quality. The higher yield was reached with an ultrasound treatment of 50 min at 40 °C and 400 W, with a pulsation interval of 2 s. Ultrasound treatment can increase the thermostability and antioxidant activity of the oil.	Sun et al. (2018)
	Ultrasound assisted extraction by sonication at 20 kHz, 75% of amplitude, 3 seconds of pulsation interval; 1, 15 and 20 min	Lyophilised and grounded <i>Bombyx mori</i> Microwave dried and grounded <i>T. molitor</i> and <i>Gryllus bimaculatus</i>	Ultrasound sonication significantly affects the protein extraction yield without changing the amino acid profile. Sonication time had a different impact on protein extraction yield among several insect species (maximum yield reaches after 15 min for <i>T. molitor</i> and <i>G. bimaculatus</i> and 5 min for <i>B. mori</i>) The protein extraction yield was dependent on the insect species and their life stage.	Choi et al. (2017)
	Ultrasound assisted protein extraction by sonication with amplitude 70% for 6 min. pulsation interval of 30 s.	Defatted powder of <i>Apis mellifera</i> larvae and pupae Defatted powder of <i>Schistocerca gregaria</i> adults	Ultrasound sonication improve the protein extraction yield Method used for protein extraction alter the molecular characteristics of protein, determining a changing on its hydrophobicity. But a specie-specific effect has to be addressed.	Mishyna et al. (2019)
	PEF at 2 and 3 kV/cm; 5, 10 and 20 kJ/kg wet basis	Live <i>H. illucens</i> larvae	Oil extraction yield was not affected significantly by PEF treatment. Higher amount of amino acids was recovered in oil after PEF treatment	Alles et al. (2020)

¹ CAPP = cold atmospheric pressure plasma; HHP = high hydrostatic pressure; LAB = lactic acid bacteria; PEF = pulsed eclectic field; TVC = total viable count; US = ultrasound; YM = yeasts and moulds.

insect species and the solvent used, with better results found for *T. molitor* oil extracted with ethanol-water mixture. Important parameters that determine the extraction yield are also temperature, treatment time, pulse interval and ultrasound power. Optimisation of ultrasound treatment in order to increase the oil extracted from *Clanis bilineata* was conducted by Sun et al. (2018). In this study, they applied Response Surface Methodology to find out the best parameters combination in order to increase the extraction yield without altering the antioxidant properties of the oil. A possible pathway where integration of ultrasound is used for insect oil extraction is shown in figure 5 (pathway A).

PEF has also been tested for oil extraction from insects (Figure 5, pathway B). In a recent study conducted on *H. illucens* larvae, Alles et al. (2020) observed that the PEF pre-treatment resulted in a size reduction and a more

homogeneous distribution of fat droplets in insect cells; however, no significant increase in the extraction yield was observed. Further, the fatty acid profile of the oil extracted from PEF pre-treated sample was observed to be similar to the oil extracted without any pre-treatment.

Ultrasound, PEF, HHP and CAPP can also have an impact on protein extraction from defatted insect meal (Figure 5, pathway C). For example Choi et al. (2017) observed an enhanced protein extraction yield on *B. mori*, *T. molitor* and *G. bimaculatus* treated when ultrasound was used, while Mishyna et al. (2019) observed that ultrasound assisted extraction can enhance solubility, coagulability and foaming stability of *S. gregaria* protein extracts, while the same treatment has been responsible for reduction in solubility and coagulability in *A. mellifera* extracts.

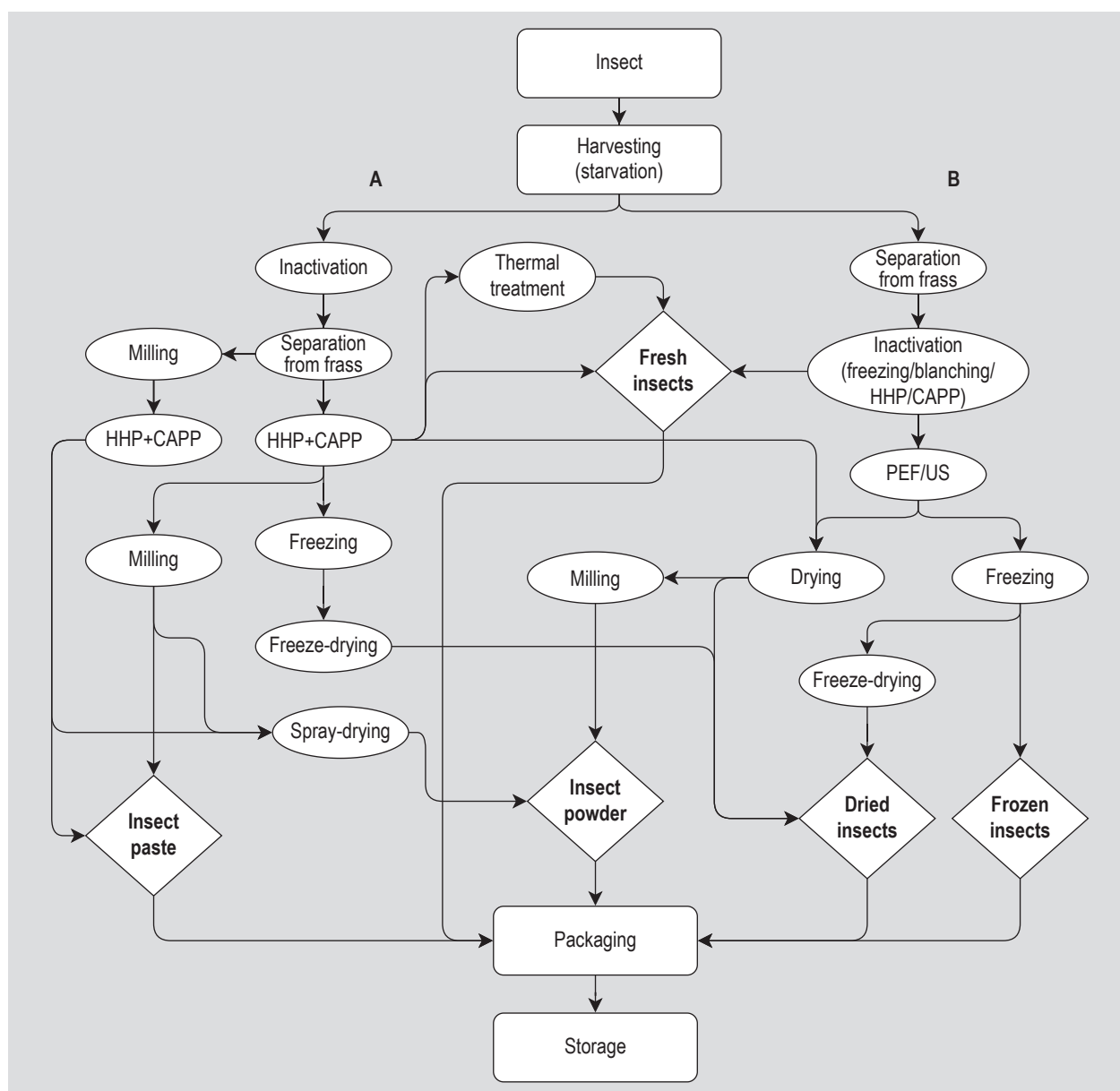


Figure 4. Possible innovative processing pathways for the production of whole (fresh/frozen/oven-dried/freeze-dried/microwave-dried) edible insects and full-fat insect meal

Enzymatic hydrolysis

Innovative food processing methods, such as microwave, pulsed-electric field, ultrasound and HHP alone or coupled with enzymatic hydrolysis can reduce protein allergenicity of food proteins including edible insect products (Dong et al., 2021). In particular to insects, HHP and ultrasound have been studied to enhance the effect of hydrolysis of these allergenic compounds in order to increase the product quality. In a recent study, Boukil *et al.* (2020) reported that HHP at 380 MPa for 1 min applied in combination with Alcalase® enzyme could improve the protein hydrolysis degree (by up to 24%), allowing a partial reduction in allergenic activity. Ultrasonic treatment can also enhance

the hydrolysis degree of insect powders (Mintah *et al.*, 2019a). For example, defatted *H. illucens* meal treated with ultrasound (600 W, frequency of 40 kHz) at 50 °C for 30 min, presented higher enzymatic activity (Mintah *et al.*, 2019a). Moreover, the damaged cells could lead to increased enzyme releases/enzyme concentrations, enhancing the hydrolysis of allergens (Mintah *et al.*, 2019a). Nevertheless, ultrasound treatment can also affect the antioxidant properties of the powder. Optimisation of the process depending on some antioxidant parameters has been conducted and validated on defatted *H. illucens* meal, concluding that sonication before the enzymatic hydrolysis allows obtaining a product with higher antioxidant power (Mintah *et al.*, 2019b). Furthermore, ultrasound can cause a change in the

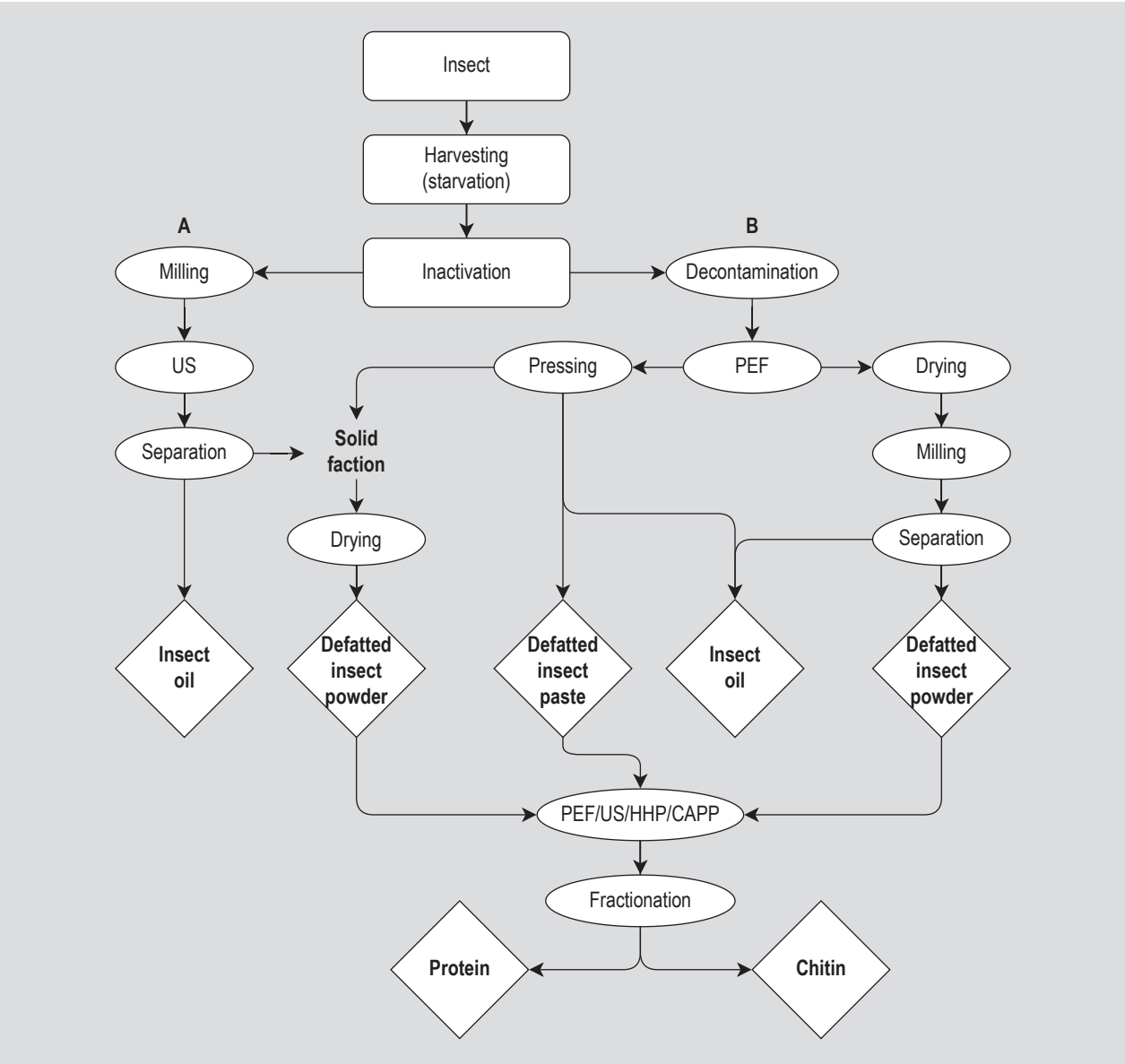


Figure 5. Possible innovative processing pathways for the production of defatted insect meal, insect oil, insect protein and chitin.

protein structure. Mintah *et al.* (2020) observed a reduction of β -sheet and a correspondent increase of α -helix and β -turn conformations. However, the proportion of each structure depends on the treatment parameters applied (Mintah *et al.*, 2020).

5. Conclusion and future perspectives

With the growing production of edible insects at an industrial scale, it is crucial to implement appropriate post-harvest and processing technologies for the safety, preservation, quality improvement, fractionation and storage of insects and insect products. Conventional processing pathways may include several unit operations used in traditional food and feed processing. The insect processing pathways

can vary depending upon the nature of the initial material and the desired end product. In recent years, novel food processing technologies (e.g. high pressure processing, PEF, ultrasound and CAPP) have shown potential as an alternative or synergistic to the conventional technologies. The main challenges ahead of insect processing are the development of efficient, environmentally friendly and low-cost processing technologies, waste minimisation, recovery, and incorporation of by-products/co-products. Novel food processing technologies are considered to be environmentally friendly and may improve the processing efficiency, safety, quality, and sustainability of insect based products. However, the high knowledge level requirement, regulations and initial plant costs represent important hurdles to overcome.

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Conflict of interest

The authors declare no conflict of interest.

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A4. Storage and packaging of edible insects

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Storage and Packaging of Edible Insects

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13.1 INTRODUCTION

13.1.1 Basic Principles of Storage

The need for food storage from the time of procurement to actual consumption has been a necessity for humans since the early hunting and gathering times. In today’s context, from farm to fork, the food passes through various stages of harvesting, processing, distribution, and retail, and at each stage, there is a need

for storage/transport for short/long durations. Due to globalization, the geographical distance between the actual production site and consumers has increased, and the time between actual production to final consumption can be very long. Thus, the need to store food for a long time while still preserving its quality to the maximum is of paramount importance. Additionally, the demand for food products all year round has laid acute emphasis on the development of various storage techniques.

The selection of the type of storage depends on the scale of production/supply of the product and the product properties. On large scales, food can be bulk stored in huge storage areas in stacked containers, or storage areas can be specifically designed for the product, e.g. silos for grains. At smaller scales and for retail distribution, small containers or packages are used. In such cases, the type of packaging and material used plays an important role, which will be discussed later.

In storage, the atmosphere (temperature, humidity and gaseous concentration) surrounding the food product can affect its quality and shelf life. Some different storage techniques based on modification of the storage atmosphere are:

Cold/refrigerated storage: Fresh food continues to undergo metabolic processes, such as the breakdown of carbohydrates, proteins, etc., into simpler compounds, even after harvest/slaughter throughout the supply chain. These metabolic processes are highly temperature-dependent (Kitinoja, 2013). Moreover, spoilage-causing microorganisms such as bacteria and fungi that may be present in food, and their activities/multiplication, are also temperature specific. Hence, temperature control is the foremost factor to be considered in the storage/transport of food substances. The temperature regulation is highly dependent on product properties; for instance, meat, fish, etc., may require frozen temperatures, while fresh fruit and vegetables may suffer freeze injuries at sub-zero temperatures.

In chilled storage, a temperature between -1°C and 10°C is maintained, whereas in frozen storage, products are stored at temperatures below -18°C (Evans et al., 2014). Another type of storage could be hyperbaric cold storage, which involves the storage of food at pressure usually below 100 MPa and at low temperatures, e.g., storage of fish (Otero, Pérez-Mateos, Holgado, Márquez-Ruiz, & López-Caballero, 2019). This technique would allow storage at sub-zero temperatures without freezing (e.g. about -10°C at 100 MPa).

Controlled atmosphere storage: Often in the storage of fresh produce, temperature control alone is not sufficient for quality maintenance of fresh produce. In such instances, a controlled atmosphere is effective. Controlled atmosphere storage involves continuous monitoring and control of a specific gaseous concentration. The specific temperature requirement, along with the most suitable oxygen and carbon dioxide concentration to minimize spoilage and maximize the shelf-life of fresh produce, varies between different produce and is also dependent on the cultivar, season, maturity and post-harvest treatment (Bishop, 1997).

13.1.2 Basic Principles of Packaging

The role of food packaging can be divided into two categories – primary and secondary (Marsh & Bugusu, 2007). The primary functions are:

- To protect the food from outside influences. Packaging acts as a barrier against external physical, chemical, and biological influences. The packaging is often designed to provide physical protection against mechanical damage and may provide cushioning against shocks and vibrations. Packaging material can also prevent changes in the chemical composition of the material due to outside influences like moisture, gases, and light e.g. opaque bottles to block out light for protection of light-sensitive materials. Lastly, it provides a barrier to prevent microorganisms, rodents, and other animals from infesting and spoiling the food.
- To contain the food and reduce food waste by shelf life extension.
- To provide the consumers with relevant information regarding the composition and nutritional value of the content.

Among the secondary roles of packaging are to provide convenience, e.g., ease of handling; traceability, e.g. barcodes enabling the company to trace back the product to the manufacturing lot; tampering/adulteration reduction, e.g. seals that clearly indicate if the package has previously been opened or not.

13.1.3 Different Types of Packaging

Modified atmosphere packaging: Modified atmosphere packaging (MAP) involves the generation of a modified atmosphere inside the package that is conducive to shelf-life prolongation of the products. Higher carbon dioxide concentration and lower oxygen concentrations (1%–5%) inside packages of fresh produce (berries, apples, pears) are known to retain fruit quality for longer. Similarly, in meat-based products, the modified atmosphere might be used to inhibit the growth of anaerobic/aerobic bacteria, depending on the product. The modified atmosphere is either introduced at the beginning (before sealing) by flushing the package or allowed to develop later after the package is sealed. Development of the final gaseous concentration inside a package atmosphere is a function of the permeability and surface area of the packaging material, leakage through seals and other defective areas, product properties (e.g. in case of fresh produce consumption of oxygen and production of CO₂), temperature which may affect the permeability, and also product properties.

Vacuum packaging: In vacuum packaging, air is removed from the package. It creates an oxygen-deficit atmosphere that inhibits oxidative reactions and the growth of aerobic spoilage organisms. This type of packaging can be considered as an extension of MAP as the atmosphere around the product is modified; furthermore, any residual oxygen is consumed by microorganisms to produce carbon dioxide (Narasimha Rao & Sachindra, 2002). Vacuum packaging alone (J.-Y. Kim & Song, 2004) or in combination with other treatments or procedures such as chitosan coating (Duran & Kahve, 2020), essential oil treatment (Kačániová et al., 2019), edible coating and extracts (Dalvandi, Almasi, Ghanbarzadeh, Hosseini, & Karimian Khosroshahi, 2020), etc. has been shown to maintain quality in poultry and other meat products.

Active and intelligent packaging: Active and intelligent packaging involves the interaction of the packaging material with the food material or the environment inside the package to elongate shelf life (Restuccia et al., 2010). In active packaging, components with some active functions such as oxygen scavenging, ethylene removal, and off-odor removal are incorporated inside the package. Active packaging includes oxygen scavengers, carbon dioxide emitter/absorbers, moisture absorbers, ethylene scavengers, ethanol emitters, flavor releasing/ absorbing systems, and antimicrobial systems (Ozdemir & Floros, 2004).

Intelligent packaging provides consumers information about the product by monitoring the packaged food or the conditions within the package (Restuccia et al., 2010). Intelligent packaging may include sensors, indicators, and radio-frequency identification (RFID) systems. Sensors are devices that can detect, record signals, and transmit information; these can be biosensors that respond to biological reactions, gas sensors that can detect specific gas molecules, or chemical sensors that can detect a particular chemical or gas through surface adsorption (Biji, Ravishankar, Mohan, & Gopal, 2015). Indicators can be freshness indicators, integrity indicators, or time-temperature indicators. RFID is a technology in which wireless sensors can gather information without human intervention (Biji et al., 2015).

13.2 STORAGE OF WHOLE INSECTS

Raw insects are characterized by high moisture and water activity (Vandeweyer, Lenaerts, Callens, & Van Campenhout, 2017), whereby they are unstable and prone to microbial spoilage and chemical degradation (Kroncke, Boschen, Woyzichowski, Demtroder, & Benning, 2018; Lenaerts, Van der Borgh, Callens, &



FIGURE 13.1 Whole fresh *Tenebrio molitor* larvae stored in atmospheric air for 8 hours at: (a) frozen (-18°C), (b) refrigerated ($+4^{\circ}\text{C}$), (c) room ($+20^{\circ}\text{C}$) temperature.

Van Campenhout, 2018). When not appropriately treated and stored, their shelf life is limited to few hours (Ssepuuya, Aringo, Mukisa, & Nakimbugwe, 2016; Ssepuuya et al., 2021). Therefore, in order to guarantee a safe and valuable product for consumers, suitable preservation technologies need to be applied.

13.2.1 Fresh (Frozen and Chilled) Insects

Fresh minimally processed edible insects should be kept in controlled temperatures throughout their entire shelf life. Freezing and chilling (refrigeration) are the two main strategies used for preserving such products.

Freezing allows immobilizing the free water in a way that it will not be available for microorganisms and enzymes. Consequently, frozen insects should be kept at low temperature ($\leq -18^{\circ}\text{C}$) for their entire shelf-life in order to prevent resuming of undesirable processes including color change (Figure 13.1) (Dossey, Tatum, & McGill, 2016; Ojha, Bussler, Psarianos, Rossi, & Schluter, 2021). Nevertheless, although microbial growth may be easily controlled by such low temperatures, the same temperatures might not always be effective in terms of quality preservation. Wessels, Azzollini, and Fogliano (2020) observed an enzymatic browning reaction on *Alphitobius diaperinus* larvae stored at -20°C for 2 months. It was attributed to the activity of phenoloxidase, an enzyme naturally present in the insect body and still active at these temperatures. Indeed, despite the fact that freezing should have reduced the water activity to a safe level, the percentage of unfrozen water was still high. This was mainly attributed to antifreeze proteins, i.e. proteins able to bind ice crystals and inhibit their growth (Wessels et al., 2020). Therefore, in order to prevent such browning, frozen storage at lower temperatures (at least -35°C) or pre-treatments able to degrade the enzymes (i.e. blanching or high hydrostatic pressure $\geq 400\text{ MPa}$) were advised (Leni, Caligiani, & Sforza, 2019; Tonnejck-Srpova, Venturini, Humblet-Hua, & Bruins, 2019; Wessels et al., 2020).

Preliminary treatments may also be conducted to lower the initial microbial count and lipid oxidation. For example, Son et al. (2016) observed that blanching could inactivate the pro- and generate antioxidant compounds, resulting in reduced oxidative stress and guaranteeing at least 10–14 days of shelf-life at $+4^{\circ}\text{C}$. Similarly, S. Y. Kim et al. (2015) observed that pan-fried *T. molitor* larvae stored for 18 days at $+4^{\circ}\text{C}$ had lower TBARS (secondary lipid oxidation products) than the same insects submitted to a freeze-drying treatment. Concerning microbial contamination, raw insects are highly contaminated substrates, whereby a post-harvest treatment should always be carried out in order to bring down the initial microbial count (Garofalo et al., 2019; Ojha et al., 2021). Indeed, refrigeration or freezing can only delay (refrigeration) or prevent (freezing) microbial growth, but will not reduce their initial number (Klunder, Wolkers-Rooijackers, Korpela, & Nout, 2012; Kurdi, Chaowiwat, Weston, & Hansawasdi, 2021; Vandeweyer, Lachi, Deruytter, Van Der Borgh, & Van Campenhout, 2021; Vandeweyer et al., 2018).

When refrigeration is applied, microbial safety should be carefully checked along the entire life of the products. For example, Son et al. (2016) observed high microbial counts in blanched insects stored at 4°C for over 6 days, reaching significant contamination after 2 weeks (Son et al., 2016). MAP has been proposed as method for controlling microbial growth in fresh foods during storage (Flekna, Mäke, Bauer, & Bauer, 2017; Stoops et al., 2015), although Van Campenhout, Lachi, and Vandeweyer (2021) did not observe any particular advantage of using vacuum packaging on refrigerated black soldier fly larvae. On the other hand, when vacuum or other oxygen-free atmospheres are used, particular attention should be given to anaerobic bacteria since they are facilitated to grow. For example, Nightingale and Ayim (1980) documented an outbreak of botulism (illness due to the ingestion of a toxin produced by the anaerobic bacteria *Clostridium botulinum*) after ingestion of ants stored in anaerobic conditions, while Ssepuuya et al. (2021) recorded the spoilage of roasted grasshopper after only 24 hours of vacuum storage.

MAP may also be employed for controlling the lipid oxidation in refrigerated whole insects, as observed in crickets kept in vacuum-filled nitrogen packaging (Gan et al., 2022). It is well known that oxygen is one of the main triggers for oxidative reactions (Johnson & Decker, 2015), therefore, removing it from the atmosphere in direct contact with the insects may be a primary solution for preventing lipid oxidation (Flekna et al., 2017; Ssepuuya et al., 2016).

Lipid oxidation might also be influenced by other factors, such as presence of antioxidants and temperature of storage (Ssepuuya et al., 2021), as reported by Tiencheu et al. (2013), who observed that larvae of *Rynchophorous phoenics* stored for 90 days at -18°C had the same peroxide value (PV) as larvae stored at +4°C for 3 days. Antioxidants can be added to raw insects during processing. Gan et al. (2022) observed that *Gryllus bimaculatus* adults fried with palm oil supplemented with natural or synthetic antioxidants showed higher lipid stability (lower free fatty acids, lower peroxide value and lower TBARS) over 150 days of storage. However, the antioxidant effect became evident only in the last 30 days of storage, since no clear differences in peroxide values and TBARS could be observed after 120 days.

13.2.2 Dried Insects

Drying aims to reduce water activity (i.e. water available for microbial growth and chemical reactions [Liceaga, 2021]) through physical removal of the water contained within the food. The resulting products should theoretically be stable for a long time at room temperature (Parniakov et al., 2021), although undesirable alteration may still happen. Indeed, despite the fact that microbial growth and non-enzymatic browning are repressed at water activity lower than 0.60 and 0.70 respectively, lipid oxidation has been observed in food at water activity lower than 0.30, slowing down at increased levels (Barden & Decker, 2016; Kroncke et al., 2018; Labuza, 1977; Parniakov et al., 2021; Ssepuuya et al., 2021; Sun et al., 2021).

Therefore, lipid oxidation, which can result in rancidity, is the main factor for long-term preservation of dry edible insects as they are particularly rich in unsaturated fatty acids (Oonincx & Finke, 2021; Rossi et al., 2022; Rumpold & Schluter, 2013; Ssepuuya et al., 2021). High lipid oxidation has been often recorded in dry insects stored at room temperature, as suggested by Ssepuuya et al. (2016), who observed that dried grasshoppers kept for two months at room temperature had the same peroxide value (PV) as dry insects stored 6 months at +4°C. Similarly, Tiencheu et al. (2013) noted that drying, especially if preceded by 20 minutes of boiling, resulted in an extremely high PV and low acidity (i.e. amount of free fatty acids), concluding that high temperature treatment and not-freezing storage can result in lipolysis and lipid oxidation of whole insects (Tiencheu et al., 2013). On the other hand, high temperature drying showed protective effects on lipid oxidation in other food since it may favorite enzymatic browning, such as the Maillard reaction, in which products are known to interact with lipid and protein (Domínguez et al., 2021; Zamora & Hidalgo, 2005).

The presence or absence of light and the gas composition of the atmosphere in direct contact with the product may play key roles in the lipid oxidation of dry whole insects as well. Ssepuuya et al. (2016; 2017) noticed higher oxidative stability for insects packaged in opaque and vacuum sealed bags rather than in transparent air-filled plastic boxes.

MAP and vacuum packaging may also be used for controlling microbial growth in stored dry insects, as tested by Ssepuuya et al. (2017), who observed that the microbial count of room temperature-stored grasshopper could be kept steady if vacuum packaging was applied. However, in this study no clear effect of vacuum packaging could be concluded, since normal air-packed dry insects were not tested. Moreover, other studies have not shown a direct effect of the absence of air on the microbial stability of dry insects, which always showed steady microbial counts (total viable counts, *Enterobacteriaceae*, endospores and fungi) during 6 months of room-temperature (Vandeweyer et al., 2018) or chilled (Ssepuuya et al., 2016) storage. However, despite the fact that dry insects might be considered microbiologically stable, microbial growth may always resumes, implying that suitable conditions should be guaranteed all over the storage time. For this reason, Adámek et al. (2018) suggested that in order to ensure the long-term storage of whole dried insects, hermetic packaging should always be applied. Indeed, contamination with fungi has been detected in several dry insects, which have been found to be particularly suitable for *Aspergillus* spp. growth and aflatoxin contamination (Kachapulula, Akello, Bandyopadhyay, & Cotty, 2018; Mpuchane et al., 2000; Mpuchane, Taligoola, & Gashe, 1996; Musundire, Osuga, Cheseto, Irungu, & Torto, 2016). The presence of fungi may also be favored by pests, such as live insects and mites (Mpuchane et al., 2000), which may contaminate dry commodities with feces, exoskeletons, and frass, and open cracks facilitating entrance of fungi (Allotey, Mpuchane, Gashe, Siame, & Teferra, 1996; Mpuchane et al., 2000; Nayak & Daglish, 2018). Controlling such infestation is, therefore, a key step for preventing deterioration in the quality and safety of stored dry insects. This operation may be conducted by using additives such as dried pepper or salt (10 grams each 30 g of dry insects), as shown by Fasunwon, Banjo, and Jemine (2011).

13.3 STORAGE OF PASTE AND POWDER DERIVED FROM INSECTS

In order to increase consumers' acceptability and/or use of insects as an ingredient in several food preparations, whole insects may be reduced in paste or powder (Modlinska, Adamczyk, Goncikowska, Maison, & Pisula, 2020; Yazici & Ozer, 2021). Milling of whole insects can be conducted on fresh as well as on dry material (Ojha et al., 2021). In the former case, it results in a paste, while if the grinding process is preceded by drying, insect flour is obtained. The two products are characterized by a completely different moisture level, whereby their stability during storage will be very different.

13.3.1 Insect Paste

The main concern during the preservation and storage of insect paste should be the microbial stability. Milling fresh insects always results in products with high microbial load. Indeed, breakage of the intestinal tract leads to a significant microbial release from the insect gut, resulting in unstable and potentially dangerous material (Grabowski & Klein, 2017; Klunder et al., 2012; Rumpold et al., 2014). This implies that, in order to ensure food safety of the product, milling operations should always be followed by processes for microbial reduction (Frohling, Bussler, Durek, & Schluter, 2020; Klunder et al., 2012; Ojha et al., 2021). These processes can have a strong impact on the quality, safety, and stability of the final product and the most appropriate storage strategy for product preservation (Elhassan, Wendin, Olsson, & Langton, 2019; Kamau et al., 2018a, 2018b; Stoops et al., 2017; Womeni et al., 2012). Therefore, since the quality of the initial material might be very different, equal storage conditions may result in different shelf-life for insect meal obtained from several species or from the same species that has undergone different treatment (Kamau et al., 2018b; Lee et al., 2020).



FIGURE 13.2 *Tenebrio molitor* paste stored for 28 days under refrigerated conditions (+4°C) by using MAP (30% CO₂ 70% N₂) after: (a) high pressure treatment (500 MPa, 5 min) or (b) blanching (40 sec), or (c) blanching and subsequent normal air packaging.

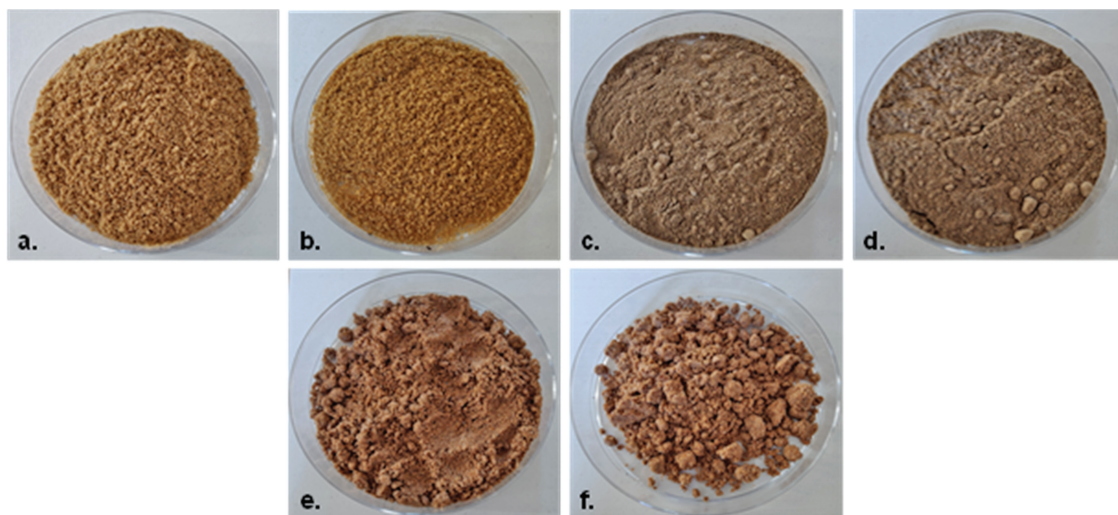


FIGURE 13.3 Insect flours stored in plastic packs at different temperatures for 1 year: *Hermetia illucens* flour (a) refrigerated (+5°C) or (b) room temperature (+20°C); defatted *Hermetia illucens* flour (c) refrigerated (+5°C) or (d) room temperature (+20°C); *Tenebrio molitor* flour (e) refrigerated (+5°C) or (f) room temperature (+20°C).

Among the most used method for preservation of fresh products, MAP along with the use of a low temperature seem to be the most appropriate strategies for insect paste preservation. For example, Stoops et al. (2017) observed slower microbial growth in insect paste packaged in 60% CO₂ and 40% N₂ rather than in normal air, while De Smet et al. (2019) noticed an absence of microbial growth in mealworm paste stored at -21°C rather than at 4°C, where microbial growth was already observed during the first week. Figure 13.2 presents the effect of storing *Tenebrio molitor* paste for 28 days under refrigerated conditions (+4°C) by using MAP compared to air.

Alternative methods may include the use of chemical or natural additives and fermentation. Chemical additives such as sodium lactate (50 g/kg of a 60% solution) and sodium nitrite (150 mg/kg) have been tested for mealworm paste preservation by De Smet et al. (2019) and Borremans, Smets, and Van Campenhout (2020). In both studies, no significant effect due to the additives was observed on total microbial count, while lower lipid stability was detected in pastes treated with the aforementioned preservatives. On the other hand, lower browning and therefore higher color stability were reached by using the additives (Borremans et al., 2020), although it was not enough to justify their use in edible insect paste preservation.

Fermentation has also been extensively studied as preservation method for insect paste. Borremans et al. (2019) showed that a wide range of microbial starters for meat fermentation are suitable for the

fermentation of insect paste, the final quality of which may be strongly impacted by the microbial community of the chosen starter. Use of such starters is a key aspect in insect paste fermentation, since it may ensure rapid and regular acidification of paste, preventing the growth of undesirable microorganisms and the production of undesirable flavor (Borremans et al., 2019; Borremans, Lenaerts, Crauwels, Lievens, & Van Campenhout, 2018; Borremans et al., 2020; Van Campenhout et al., 2021). Nevertheless, the use of nitrite or other additives before fermentation is still advised since sulphite-reducing bacteria can easily grow in fermented pastes, generating the unpleasant odor of rotten eggs (De Smet et al., 2019).

13.3.2 Insect Powder

Unlike insect pastes, dry insect flour can be considered relatively stable from the microbial point of view, but it is still very susceptible to chemical degradation and color change (Figure 13.3) (De Smet et al., 2019; Klunder et al., 2012; Lee et al., 2020; Ssepuuya et al., 2021). For example, cricked powder stored for 18 months at high temperatures (25°C, 35°C and 40°C) did not show any significant microbial contamination (D. H. Kim et al., 2016), while lipid oxidation with a fast increase in peroxide and *p*-Anisidine values were detected after 45 days of room-temperature storage (Kamau et al., 2020; Kamau et al., 2018a).

Strategies for reducing or preventing lipid oxidation in insect powder might be related to low temperature storage as well as the use of MAP (McMillin, 2008) or antioxidant directly or indirectly (i.e. through controlled release from the packaging) added to the product (Cuong & Chin, 2016; Doolaege et al., 2012; Rangaraj, Rambabu, Banat, & Mittal, 2021). While the last two strategies have not yet been tested on edible insect dry flour, low temperatures (4°C) have been shown to be effective in lipid oxidation reduction (Kamau et al., 2020; Kamau et al., 2018a). However, when chilled conditions are used for dry products, microbial growth should be considered. Indeed, the high humidity in refrigerators may lead to fast microbial growth (Kouřil, Burdová, & Kalhotka, 2022), so room-temperature storage is preferred.

The microbial and chemical stability of insect meal may also depend upon the packaging material used. Kamau et al. (2020; 2018a) observed that cricket powder, as well as black soldier fly powder stored for 6 months in polypropylene bags (PP), polyethylene bags (PE), and polypropylene bottles with lid (PL) showed different microbial growth, lipid oxidation, and chemical degradation ratio. It was attributed to the different gas and water vapor permeability of the tested packages, which followed the order PP<PE<PL. However, microbial safety and low oxidation were always guaranteed whether room temperature storage was applied or not (Kamau et al., 2020; Kamau et al., 2018a).

13.4 STORAGE OF INSECT FRACTIONS

Insects may possess distinct physical and sensory characteristics that make them unattractive as food, especially in Western cultures where they are not consumed traditionally. Therefore, methods for extraction of insect fractions (proteins and lipids) for incorporation into other conventional foods can increase their acceptance. Little is known about the storage and its effect on the functionality of insect-based food ingredients; therefore, this is a potentially important area for research and technological innovation. The storage requirements can differ based on the form of the ingredients, which may include powders, pastes, liquids, and so forth.

13.4.1 Protein Powder

Production of protein-rich flour from insects for incorporation into foods provides an opportunity for insect protein to achieve widespread use directly in human diets. Therefore, there is a growing interest

in generating protein isolates and concentrate from edible insects (Ndiritu, Kinyuru, Kenji, & Gichuhi, 2017). In general, dried protein powders are characterized by a low moisture content and water activity and thus are not particularly susceptible to microbiological spoilage through the growth of bacteria, yeasts, and molds. Their spoilage is mainly caused by physical (changes in flowability, solubility, water activity, and color) and chemical processes (protein oxidation and denaturation). Optimum packaging and storage conditions are essential in maintaining functional properties and other desired quality attributes in dried insect protein powder. Extrinsic factors, mainly humidity and temperature, are the most important and controllable factors for delaying degradation of food protein powders during storage. For example, ideal storage conditions for dry dairy products are temperatures below 25°C with a relative humidity below 65%, which can also be replicated for insect protein powders. The greatest loss of product quality during storage is often the result of moisture uptake by the powders, which may result in chemical, physical, functional, and bacteriological changes. Protein solubility, water binding, oil binding, gelation, emulsification, and foaming are the important functional properties of proteins, and these are measured to determine the behavior of proteins in different food applications. The storage time, humidity, and temperature differences can affect the water activity and solubility of protein powders. For example, Murayama, Zhu, and Ikeda (2021) reported that the solubility of high-protein milk protein concentrate may decrease significantly during storage, particularly at relatively high temperatures and humidity.

13.4.2 Insect Oil

Lipids are the second-largest nutritional fraction of insects, and their content is higher in the larval stage of the insects' life. Insect lipids can interfere with protein extraction; therefore, lipid separation is generally carried out to enhance protein extraction. The lipid fraction of insects is a mix of triglycerides and saturated and unsaturated fatty acids, with variation depending upon the species, diet, life stage, and environment. During storage, all oils and fats oxidize and/or hydrolyze, resulting in rancidity and compromised nutritional profiles. The rate of oxidation depends mainly on several intrinsic factors including fatty acid profile, minor components like moisture, and other impurities as well as external factors including air (oxygen) concentration, light exposure, and temperature during storage. Mealworm and black soldier fly oil have been reported to exhibit a superior oxidative stability than vegetable oils (Jeon et al., 2016; Matthäus, Piofczyk, Katz, & Pudiel, 2019). Matthäus et al. (2019) suggested the high oxidative stability of *H. illucens* larvae fat due to the high amount of saturated fatty acids, which show a higher oxidative stability than unsaturated fatty acids. Depending on the quantity, storage time, and end use, insect lipids could be stored in drums, cartons lined with cans, and glass jars.

13.5 PACKAGING AND STORAGE OF INSECT-BASED FOOD PRODUCTS

Several producers in Western countries are producing a number of insect-based processed food products such as protein powders, energy bars, bread, cereals, crackers, pasta, and snacks, as well as burgers and meatballs. Normally, the insect component is combined with other food materials to form the product; therefore, the packaging and storage requirements remain similar to conventional products. For example, similar to meat burgers, insect-based burgers are prone to microbial spoilage; therefore, they may be packaged under MAP or vacuum and kept in refrigerated or frozen storage. For products having low water activity, such as cereals, crackers, and other snacks, it is important to consider breakage, resulting in an aesthetically undesirable product, moisture gain resulting in loss of crispness, lipid oxidation resulting in rancidity and off-flavors, and loss of nutrients and flavor while selecting suitable packaging materials.

13.5.1 Materials Used for Packaging

Plastics: Conventionally used plastic materials are polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS). Plastics are lightweight materials that have half the density of glass and about the same density as paper. Additionally, very thin plastic films that are strong, water resistant, and have better barrier properties than paper can be made. Hence, plastic packaging consumes a lot less resources and it is more efficient when it comes to transportation. Due to their low costs, plastics are often preferred in food packaging.

Composite materials: Different packaging layers to provide required barrier properties or to better retain quality may often be used. In a study (Vimercati et al., 2020), ready-to-eat pasta enriched with different ingredients such as spinach, beet, ginger, etc. was stored in transparent plastic packaging (inner layer polyethylene and outer layer polyester) and in metallic packaging (inner layer polyethylene and outer layer metallic polyester). The metallic packaging showed better results in color preservation in the pasta.

Cans: Cans are often used for carbonated drinks and storage of food products. These cans are intended for long-term shelf storage, and the food cans are generally subjected to a heating process. On cooling, a vacuum is generated inside the can, which minimizes food spoilage (Page, Edwards, & May, 2003).

Glass bottles: Due to its inert nature as well as its reusability, glass is a suitable material for food packaging. Glass containers can be used to store a variety of products ranging from powders and processed foods to liquids, as well as heat-treated or carbonated products packed under pressure. Some examples are processed fruit and vegetables, fish and meat, jams, marmalade, instant coffee, baby food, wine, beers, liquors etc.

Cardboard and paper-based packaging: Cardboard and paper-based packaging form an integral part of food packaging and represent the highest share (48%) of the total food packaging market (Fadiji, Berry, Coetzee, & Opara, 2018). Paper and cardboard-based materials are also widely used in the packaging of food products. With direct contact, these materials are used for the packaging of dry products such as grains, e.g., flour, rice, noodles, and breadcrumbs. With an internal coating, these packaging materials are used in the packaging of frozen food products such as vegetables (Ewender, Franz, & Welle, 2013).

The use of paper and cardboard-based packaging can have several advantages over plastic packaging. Plastic-based packaging often is easy to crack and bend, it has a low heat resistance, and laminates are costly, whereas corrugated paper packaging such as corrugated cardboard is lightweight, has good mechanical and anti-shock properties, is easy to transport, and has better heat resistance compared to plastics, as well as being printable (Kaushal, Sirohiya, & Rathore, 2015). Often paper and cardboard-based packaging materials provide a recyclable solution in comparison to many plastic-based packaging types.

13.5.2 Examples of Packed Insect-Based Food Products

As mentioned earlier, in general, insect-based food products require similar packaging as conventional products. For example, the best packaging for dried insect powder should minimize air and moisture entry into the product. Packaging for dried insect protein isolates may be adapted from other protein powders. Requirements for oxygen transmission rate (OTR) and water vapor transmission rate (WVTR) can play a vital role due to high surface area/product ratio. Consequently, metal or composite cans or aluminum-laminated flexible films have been proposed and tested. Guo et al. (2015) used polyethylene (terephthalate/nylon/aluminum/polyethylene) bags (O_2 permeability of 0.024 mL m^{-2} after 24 h at 0.1 mPa at 25°C ; water permeability of 0.006 g m^{-2} after 24 h at RH 90 % and 40°C) for storage of soy protein isolates. Shakeel-Ur-Rehman, Farkye, and Schaffner (2003) found similar performance for kraft paper bags and LDPE/PP bags for skim milk powder storage at 37°C and 90% relative humidity. Further, vacuum or inert gas packaging can be an attractive packaging strategy to maintain the quality of insect protein isolates for longer periods, especially at lower temperatures. For example, vacuum-packed fish protein

TABLE 13.1 Overview of the main spoilage mechanisms, factors to be considered during storage, and recommended packaging and storage solutions for selected edible insect–derived products

PRODUCT	SPOILAGE MECHANISMS			IMPORTANT STORAGE FACTORS	PACKAGING AND STORAGE SOLUTIONS
	MICROBIAL	BROWNING	OXIDATION		
Fresh insects	+++	++	-	Temperature	Freezing; refrigeration in MAP with low oxygen
Dry insects	-	+/-	+++	Oxygen, light, temperature	Refrigeration in opaque bag; room temperature in vacuum-hermetically sealed packaging
Entire dry flour	+	++	+++	Humidity, oxygen, light, temperature	Room temperature in MAP with low oxygen
Defatted dry flour	+	-	+++	Humidity, temperature	Freezing; refrigeration in MAP with low oxygen or vacuum-hermetically sealed packaging
Insect paste	+++	++	-	Temperature	Freezing; refrigeration in MAP with low oxygen level
Insect oil	-	-	+++	Light, oxygen, temperature	Refrigeration in MAP with low oxygen level and opaque bag

powder maintains its properties for up to 9 and 6 months at -18°C and 5°C respectively; however, it loses them rapidly at 30°C (Carvajal, Lanier, & MacDonald, 2005; Shaviklo, 2015). Table 13.1 presents some examples of possible packaging and storage solutions for insect-based foods.

13.6 FURTHER CHALLENGES AND PERSPECTIVES

Apart from basic functions of protection and preservation, packaging can serve as a mode of communication to consumers. Many Western consumers are often willing to try insect-based foods, but integrating insects into regular diets in the long term is still considered challenging (Kauppi, Pettersen, & Boks, 2019). To achieve regular consumption of insect-based foods, several strategies can be considered. In this context, the packaging of insect-based products could also play a major role. For example, the nutritional and environmental benefits of insects as food are often emphasized on packaging to specifically target health and environment-conscious consumers. Many companies use packaging design strategies to normalize insect product consumption by linking the insect products with conventional foods (Figure 13.4). In this way, these products are not categorized as an authentic or exotic insect food, but rather as recognizable Western food with added insect ingredients (Kauppi et al., 2019).

Currently, there is a growing consumer shift towards the use of more sustainable packaging. Governments are also contributing to this shift. In the EU, a circular economy has been introduced according to which all plastic packaging placed on the EU market must be either reusable or can be recycled in a cost-effective manner by 2030 (European Commission, 2018). However, a future packaging concept should support resilient food system strategies including circular processing using chitosan (e.g. from edible insects) and other bio-composites as packaging materials.



FIGURE 13.4 Examples of commercially available insect-based consumer food products in Germany: (a) freeze-dried insects by Snack Insects; (b) insect burger by Bug Foundation; (c) insect pasta by Plumentofoods; (d) crackers from Pinaks; and (e) insect granola by Snack Insects. (Adapted from Orsi, Voege, & Stranieri, 2019.)

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A5. Fluorescence-based characterisation of selected edible insect species:
Excitation emission matrix (EEM) and parallel factor (PARAFAC) analysis

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Fluorescence-based characterisation of selected edible insect species: Excitation emission matrix (EEM) and parallel factor (PARAFAC) analysis

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ABSTRACT

Fluorescence spectroscopy coupled with chemometric tools is a powerful analytical method, largely used for rapid food quality and safety evaluations. However, its potential has not yet been explored in the novel food sector. In the present study, excitation emission matrices (EEMs) of 15 insect powders produced by milling insects belonging to 5 Orthoptera species (*Acheta domesticus*, *Gryllus assimilis*, *Gryllus bimaculatus*, *Locusta migratoria*, *Schistocerca gregaria*) from 3 different origins were investigated. Parallel factor (PARAFAC) analysis performed on the overall averaged dataset was validated for five components, highlighting the presence of five different fluorescence peaks. The presence of these peaks was confirmed on each species, suggesting that fluorescence compounds of edible insects are the same in several species. PARAFAC analysis performed on the overall averaged dataset after alternatively adding the EEM recorded from one standard compound allowed to speculate that edible insects fluorescence raises from mixtures of: tryptophan + tyrosine (PARAFAC component-1), tryptophan + tyrosine + tocopherol (PARAFAC component-2), collagen + pyridoxine + pterins (PARAFAC component-3). This study suggests that fluorescence spectroscopy may represent a powerful method for investigating composition and quality of insect-based foods.

1. Introduction

Edible insects represent a very interesting trend in the food sector as they can grow on waste and by-products, converting them into materials rich in nutrients (Bosch et al., 2019; Gasco et al., 2020; Ojha et al., 2020; Pinotti et al., 2019). As their consumption spreads in developed countries, an increasing number of companies show interest in their production and transformation. However, due to the particular nature of this new food, accurate quality evaluation should always be performed in order to prevent unexpected quality changes for the consumers and economic and legal issues for the producers.

Traditional analytical techniques are destructive, time-consuming and need strong knowledge support (Hassoun et al., 2020), making them difficult for industrial applications. Innovative, fast and alternative methods include spectroscopic tools such as fluorescence spectroscopy, (Fourier Transformed) infrared spectroscopy, X-ray spectroscopy, Raman spectroscopy, nuclear magnetic resonance and others. These are characterised by rapidity and no need of sample preparation, making them suitable for in-line quality monitoring (Andersen and Mortensen,

2008; He and Sun, 2015; Li and Church, 2014; Patra, 2003; Porep et al., 2015; Silva et al., 2020). Among these, fluorescence is a very attractive technique because it appears to be 100–1000 times more sensitive than other spectroscopic methods (Albani, 2012; Strasburg and Ludescher, 1995), allowing a wide range of analyses in short time (Hassoun et al., 2020). Fluorescence landscape, also known as optical fingerprint or excitation emission matrix (EEM) is a tridimensional representation (i.e. three-way array) of the fluorescence spectra of one sample, which is recorded over several excitation and emission wavelengths. It describes the overall fluorescence pattern of the sample, allowing to perform a comprehensive chemical and physical characterisation of the product (Andrade-Eiroa et al., 2013). However, due to the extreme complexity of biological samples, along with some phenomena as quenching and interaction among several compounds, interpretation of EEM may be challenging (Elcoroaristizabal et al., 2015), requiring the use of powerful chemometric tools, such as PARALLEL FACTOR (PARAFAC) analysis (Andrade-Eiroa et al., 2013; Lenhardt et al., 2015).

PARAFAC is a decomposition method based on alternating least square (ALS) algorithm, which allows to visualise the EEM as the sum of

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several fluorescence compounds (fluorophores) present in the analysed sample (Bro, 1997). It has already been used to characterise complex mixtures and discriminate several biological matrices, as well as foods and drinks (Acković et al., 2018; Lenhardt et al., 2017; Sikorska et al., 2019; Stedmon and Bro, 2008; Stedmon and Markager, 2005; Wunsch et al., 2017; Yamashita et al., 2008). However, its potential is still poorly explored in the novel food sector (Khan et al., 2019; Millie et al., 2002).

Therefore, the aim of this study was to characterise the recorded fluorescence landscape of 15 edible insect powders from 5 different species. PARAFAC function was implemented on overall and intraspecies EEMs and further identification of the fluorescence compounds was performed.

2. Materials and methods

2.1. Insects

Live adult insects from 5 Orthoptera species, three belonging to Ensifera suborder (*Acheta domesticus* L., *Gryllus assimilis* L., *Gryllus bimaculatus* D.G.) and two to Caelifera suborder (*Locusta migratoria* L., *Schistocerca gregaria* F.), were purchased from 3 local shops: Feeders and More GmbH (Au in der Hallertau, Germany), ProInsects GmbH (Minden, Germany), Tropic Shop (Nordhorn, Germany).

Live insects were separated from their feed by sieving and kept at their optimal growth conditions for 24 h in order to empty their gut. Temperature and humidity were selected according to data given in literature (Table 1). Subsequently, insects were inactivated by chilling at 4 °C for 1 h followed by freezing at –20 °C for 1 day and stored at –30 °C. Frozen samples were freeze-dried (Alpha 1–4 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 3 days at –58 °C and 600 mbar and ground with a knife mill (Grindomix GM200, Retsch GmbH, Haan, Germany) at 10 rpm, applying 2 cycles of 30 s, separated by a break of 30 s to homogenize the bulk. Afterwards, insect powders were stored at –20 °C until fluorescence analyses.

2.2. Chemicals

Based on fluorescence peaks recorded from the insect powders, fourteen chemical compounds were selected and investigated (Table 2). Pure standards ($\geq 98\%$) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and Carl Roth GmbH (Karlsruhe, Germany) and used without further purification. Albumin, collagen, NADH, para-aminobenzoic acid, riboflavin, thiamin and tyrosine were analysed as powder in front-face mode. Stock solutions (1 g/L) of cobalamin, pterins, pyridoxine and tryptophan were prepared by singularly dissolving each standard in Milli-Q water, while retinol, tocopherol and tyramine were dissolved in ethanol (HPLC degree $\geq 99.5\%$; Carl Roth GmbH). Additionally, in order to investigate quenching and changes in fluorescence peak due to the interaction between compounds, mixtures of tryptophan (10 mg/l) and tyrosine (1 g/l) dissolved in Milli-Q water,

Table 1

Environmental conditions (temperature, relative humidity and photoperiods) used for fasting (24 h) of the studied insect species.

Species	Temperature (°C)	Relative humidity (%)	Photoperiods (hours; L:D)	Reference
<i>Acheta domesticus</i>	29 ± 1	65 ± 5	12:12	Oonincx et al. (2015)
<i>Gryllus assimilis</i>	27 ± 1	65 ± 5	12:12	Bertram and Rook (2011)
<i>Gryllus bimaculatus</i>	27 ± 1	60 ± 10	8:16	Song et al. (2016)
<i>Locusta migratoria</i>	30 ± 2	75 ± 5	16:8	Tu et al. (2012)
<i>Schistocerca gregaria</i>	30 ± 2	50 ± 5	12:12	Maeno et al. (2020)

Table 2

Chemical compounds and position of their maximum fluorescence peak selected for investigation of edible insect fluorescence landscape.

Chemical compound	Fluorescence Peak ($\lambda_{ex}/\lambda_{em}$; nm)	References
Collagen	335/390; 370/460	Egelandsdal et al. (2005)
Pterins	345/425	Abels and Ludescher (2003)
Retinol	325/470	Duggan et al. (1957)
Tryptophan	280/350; 295/350	Moller and Denicola (2002)
Tyrosine	270/320; 275/300	Poveda et al. (2003); Yang et al. (2017)
Albumin	280/340	Bi et al. (2005)
Tocopherol	290/330	Zandomeneghi et al. (2005)
Thiamin (vit. B1)	370/460	Yang et al. (2016)
Riboflavin (vit. B2)	270/525; 370/525; 450/525	Yang et al. (2016)
Pyridoxin (vit. B6)	250/370; 325/370	Yang et al. (2016)
Cobalamin (vit. B12)	250/337	Pourreza et al. (2017)
NADH	335/414–438	Dufour et al. (2003)
p-aminobenzoic acid	295/345	Duggan et al. (1957)
Tyramine	275/310	Duggan et al. (1957)

tryptophan (0.1 g/l) and tocopherol (1 g/l) dissolved in ethanol, tyrosine (1 g/l) and tocopherol (1 g/l) dissolved in ethanol, tryptophan (0.1 g/l), tyrosine (0.7 g/l) and tocopherol (0.07 g/l) dissolved in ethanol, were prepared.

After dissolving, each solution was homogenized by stirring for 60 min at 1000 rpm. Subsequently, individual operative solutions with a concentration ranged between 10 mg/L and 1 g/L were prepared. Each solution was stored at 5 °C for 48 h prior to fluorescence analysis.

2.3. Fluorescence measurement

Fluorescence analyses of powdered insects and standards were carried out with a Perkin-Elmer LS55 Luminescence Spectrometer (Perkin-Elmer, Waltham, MA, USA) controlled by FL WinLab Perkin-Elmer 403 software. A pulsed xenon lamp set at 770 V and a red-sensitive photomultiplier (R928) were used as excitation source and emission detector, respectively. Insect powders, as well as powders of standard compounds, were analysed in front-face mode (Cell-holder nr. 52123130, Perkin-Elmer) using the multi-scan application and an incident angle of 60°. The illuminated area of the front-face cuvette was 7 mm high and 3 mm wide. Analyses of standard solutions were performed in 1 mm path-length acrylic transparent cuvettes (Sarstedt AG & Co., Nümbrecht, Germany) by using the standard single position Cell-holder (L2250140, Perkin-Elmer). Analysis of solvents (blank) was performed with the same setting and using the same cuvettes before the standard solutions were analysed. Insect powders were allowed to reach room temperature in the dark for 12 h before the analysis.

Fluorescence landscapes were obtained by scanning the emission spectra from 260 to 700 nm with steps of 1 nm, over excitation ranged between 240 and 600 nm in 5 nm steps. Excitation and emission monochromator slit widths were set at 10 nm and 2.5 nm, respectively. FGL280S Long-pass filter (Thorlabs, Newton, NJ, USA) was used as emission filter, while FGUV5S Band-pass filter (Thorlabs, Newton, NJ, USA) and FGS900S Long-pass filter (Thorlabs, Newton, NJ, USA) were the excitation filters used within the excitation interval 240–345 nm and 350–600 nm, respectively. All the measurements were performed at room temperature (20 ± 2 °C) and with an integration time of 0.06 s (scan speed = 500 nm/min). The data detected were mean-centred, block averaged, rationed and corrected for rhodamine reference cell. Each sample was scanned in triplicate and three independent spectra for each sample were obtained.

Raman peak was recorded by scanning Milli-Q water contained in a 10 mm cuvette at excitation 350 nm and emission ranged between 370 and 500 nm. Raman scan was performed in triplicate, and the three

scans were mediated.

2.4. Data pre-processing

Recorded data (Raman spectra, samples spectra and blank) were read in Microsoft Excel (2010) (Microsoft Corporation, Albuquerque, NM, USA) and organized in order to have the overall spectra for each sample in one file.

In order to remove the specifics of the instrument and obtain correct, reproducible and easier to analyse EEMs, data were pre-processed through filter correction, Raman normalization and Rayleigh scatter deletion. Moreover, blank subtraction and inner filter effect correction were performed on filter corrected data for standard solutions in order to consider and delete the signals due to the solvents.

Filter correction was carried out by multiplying the recorded value with the percentage of transmission provided by the filters' manufacturer. Raman normalization was performed by dividing the fluorescence value through the area under the Raman peak, computed by integrating the filter corrected fluorescence signal recorded on Milli-Q water at excitation of 350 nm and emission between 371 and 428 nm (Lawaetz and Stedmon, 2009). Rayleigh first and second order scatter removal was performed by deleting the values at emission = excitation ± 20 nm (Rayleigh first order scatter) and emission = $[2 \times \text{excitation}] \pm 20$ nm (Rayleigh second order scatter). Deleted data at Rayleigh first order scatter were treated as missing value (Andersen and Bro, 2003), while data at the Rayleigh second order scatter were replaced by interpolation (Elcoroaristizabal et al., 2015). Finally, scatter removed matrices were reduced by deleting noisy data and redundant information as addressed by plotting the EEMs. Therefore, data recorded at excitation higher than 530 nm and emission higher than 550 nm were deleted (Bugden et al., 2008).

Inner filter effect correction was performed for standard solutions by multiplying the EEMs by a correction matrix, which was calculated for each wavelength pair from the sample absorbance, recorded in triplicate with a Perkin-Elmer Lambda 950 UV-VIS/NIR spectrometer by assuming excitation and emission pathlengths of 0.5 cm in a 10 mm quartz cuvette (Ohno, 2002).

Filter correction, blank subtraction and inner filter correction were performed on Microsoft Excel 2010, while Raman normalization and Rayleigh scatter removal were carried out on MatLab R2019a (The MathWorks Inc., Natick, MA, USA), using drEEM toolbox version 0.6.3 (Murphy et al., 2013).

2.5. Chemometric analysis

PARAFAC analyses were implemented on the corrected data by using drEEM toolbox (Murphy et al., 2013), version 0.6.3, run under MatLab R2019a (The MathWorks Inc., Natick, MA, USA) statistical environment. Different datasets were prepared and submitted to the PARAFAC function. In a first step, each species was analysed separately in order to address potential differences between species. Afterwards, the three replicates from the same sample were averaged and PARAFAC function was implemented on the overall averaged dataset, consisting of 15 independent spectra (5 species $\times$ 3 shops). In this way, an overview of the complete EEMs of several insects belonging to the Orthoptera order could be obtained. In a further step, the three EEMs recorded on each standard solution were averaged and studied. Identification and attribution of each peak was performed by evaluating the improvements of the PARAFAC models built on the overall averaged dataset after alternating adding the EEM recorded by each standard compound.

If the insect powders are containing the studied standard compounds, core consistency and percentage of explained variance of the new PARAFAC models should not be significantly different when compared to the ones of the PARAFAC models computed on the original dataset.

Before running the PARAFAC function, each dataset was scaled on

sample mode and normalized at unit of variance. Nine PARAFAC models, with a number of components ranged between 2 and 10, were built on each dataset by using the non-negative constraints on excitation and emission modes (Andersen and Bro, 2003). Convergence criteria, number of replications calculated to fix the models and maximum number of iterations were set at 10^{-6} , 10 and 2500, respectively. Core consistency diagnostic, percentage of explained variance and split-half analysis in 3 alternating and random splits were used to select and validate the right model (Stedmon and Bro, 2008).

3. Results

3.1. Fluorescence landscape

The corrected and reduced fluorescence landscape (excitation 240–530 nm; emission 260–550 nm), obtained by averaging three independent scans of the same insect powder, is shown in Fig. 1.

Each EEM is relative to one species purchased from one German seller. In every sample, a strong peak was detected in the region at excitation about 290 nm and emission about 330 nm. The intensity of this peak was highly variable among species and origins. Orthoptera suborder Ensifera (*Acheta domesticus*, *Gryllus assimilis*, *Gryllus bimaculatus*) displayed higher peak than Orthoptera suborder Caelifera (*Locusta migratoria*, *Schistocerca gregaria*), independently by the shop where the insects were bought. By considering several sellers, higher peak was detected on insects purchased by Feeders and More GmbH for all the studied species.

A prominent peak at $\lambda_{\text{ex/em}}$ around 340/430 nm was detected on *Locusta migratoria* from Tropic Shop, although the same peak could also be observed in the other samples. However, the aforementioned peak was not evident in samples other than *Locusta migratoria*. In all the samples, a clear peak was detected around excitation 340 nm and emission 395–400 nm. This peak was particularly evident in *Acheta domesticus* from Feeders and More GmbH. It was high in the other samples as well, although it was lower than the peak 340/430 nm ($\lambda_{\text{ex/em}}$) in *Gryllus bimaculatus* from Feeders and More GmbH. Further, a sharp peak was observed at $\lambda_{\text{ex/em}}$ of 355/460 nm in *Gryllus bimaculatus* from ProInsects GmbH, while powder of *Locusta migratoria* from the same shop was characterised by a peak at 355/420 nm ($\lambda_{\text{ex/em}}$). In powders of *Acheta domesticus*, *Gryllus assimilis* and *Locusta migratoria* from ProInsects GmbH and Tropic Shop, a weak peak was detected in the region 385–390/440–460 nm ($\lambda_{\text{ex/em}}$), while a peak at 335/390 nm ($\lambda_{\text{ex/em}}$) was noticed in *Schistocerca gregaria* from Feeders and More GmbH.

3.2. PARAFAC analysis

Core consistency and percentage of explained variance of PARAFAC models built on the averaged data recorded on the insect powders are displayed in Fig. 2. Core consistency shows a uniform decreasing trend, with a rapid fall between 4 (core consistency = 92.06%) and 5 components (core consistency = 67.91%) and between 6 (core consistency = 40.31%) and 7 components (core consistency = 7.56%). According to the core consistency, the percentage of explained variance raises from 96.54% (model-2) to 99.90% (model-10), with an important increment from 2 to 3 components (98.44%) and from 3 to 4 components (99.38%). Smaller increments were detected between 4, 5 (99.70%) and 6 (99.76%) components. Split-half analysis performed on the PARAFAC model with 4 and 5 components was validated on all the comparisons, while the same analysis carried out on model-6 was not validated (supplementary material S1).

Loadings of each component of the PARAFAC models 4, 5 and 6 described on sample, excitation and emission modes, are displayed in Fig. 3. Fluorescence peaks centred at $\lambda_{\text{ex/em}}$ 295/300 nm with shoulder at 333 nm (component blue), 285/333 nm (component orange) and 340/390–400 nm with shoulder at 420–425 nm (component yellow) could be observed in models 4 and 5 (Fig. 3b, c, 3e, 3f). Additionally,

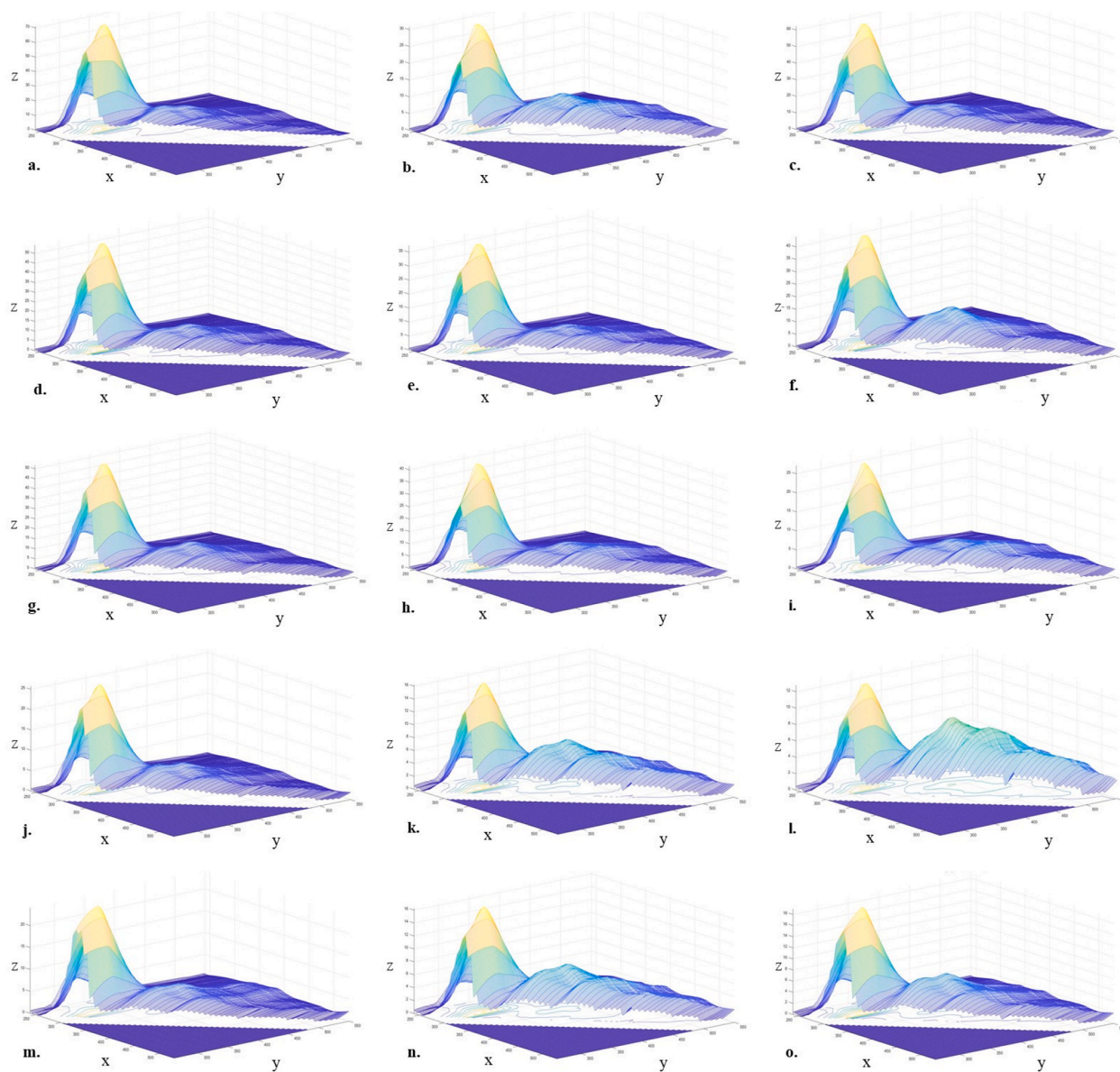


Fig. 1. Reduced, averaged and corrected fluorescence landscape recorded from the analysed samples: *Acheta domesticus* from: Feeders and More GmbH (a), ProInsects GmbH (b), Tropic Shop (c); *Gryllus assimilis* from: Feeders and More GmbH (d), ProInsects GmbH (e), Tropic Shop (f); *Gryllus bimaculatus* from: Feeders and More GmbH (g), ProInsects GmbH (h), Tropic Shop (i); *Locusta migratoria* from: Feeders and More GmbH (j), ProInsects GmbH (k), Tropic Shop (l); *Schistocerca gregaria* from: Feeders and More GmbH (m), ProInsects GmbH (n), Tropic Shop (o). X axis, excitation (nm); Y axis, emission (nm); Z axis, fluorescence intensity (A.U., arbitrary units).

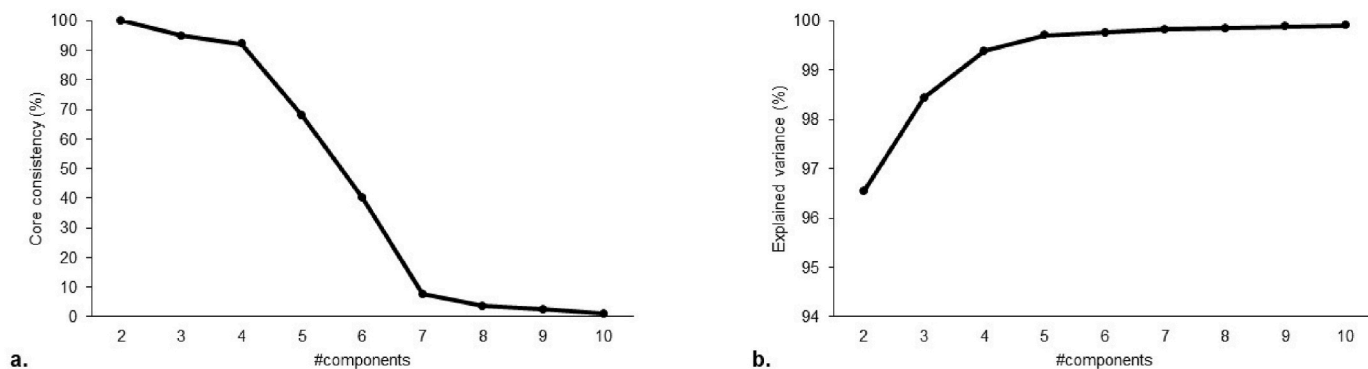


Fig. 2. Core consistency (a) and percentage of explained variance (b) of PARAFAC models from 2 to 10 components, built on the overall averaged dataset of EEM recorded from 15 different edible insect powders.

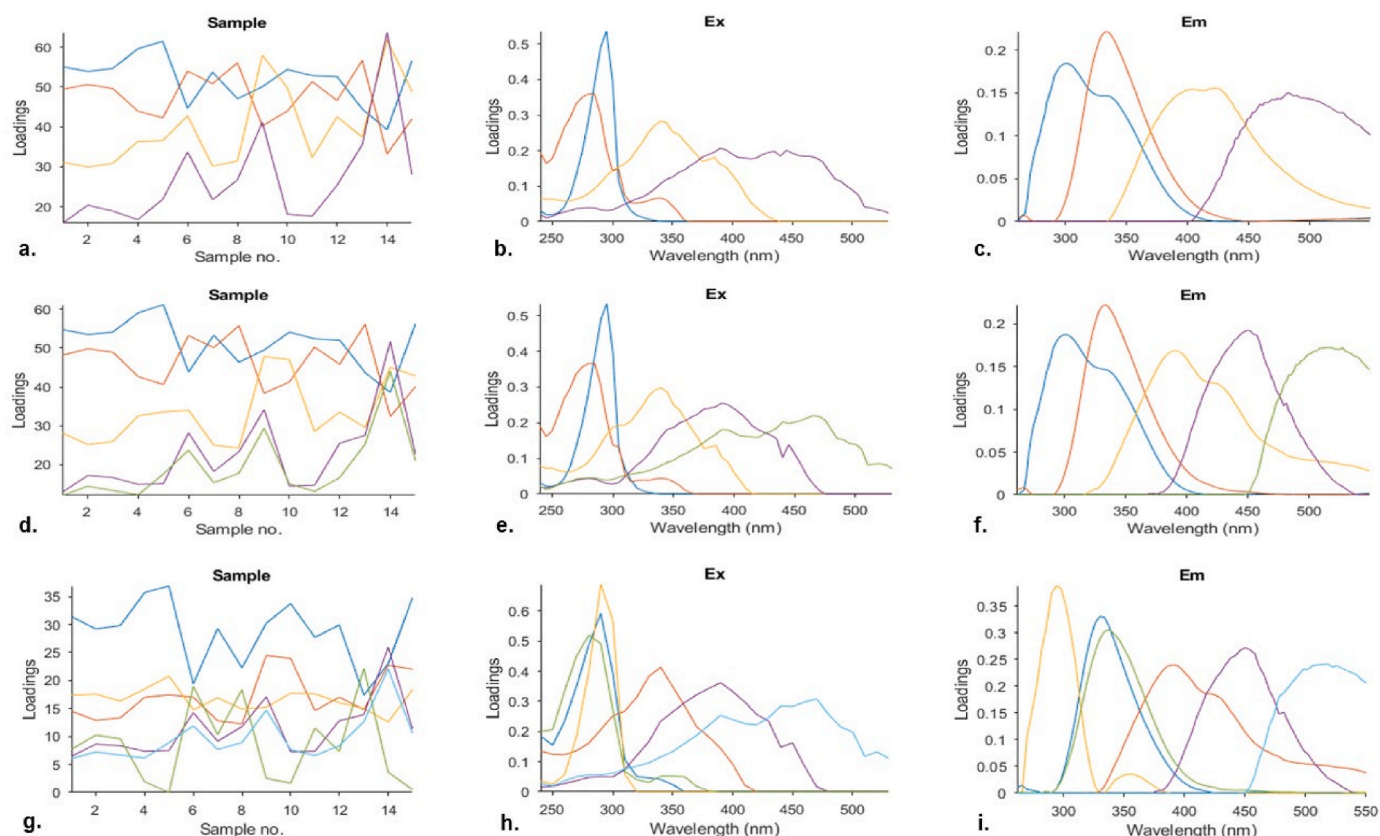


Fig. 3. Loadings of PARAFAC models 4, 5, 6 computed on the overall averaged insect EEM: model-4 (sample mode: a; excitation mode: b; emission mode: c), model-5 (sample mode: d; excitation mode: e; emission mode: f) and model-6 (sample mode: g; excitation mode: h; emission mode: i).

model-5 (Fig. 3e and f) showed clear peak at excitation 390 nm and emission 450 nm (component violet). The same component was also detected in model-4; however in this model, it displayed a broader range of excitation, with maximum at 390 nm and shoulder between 430 nm and 470 nm, while the emission was ranged between 458 and 527 nm. A further component (green) with excitation maxima at 465 nm and shoulders at 390 nm and 520 nm, corresponding with emission ranged between 504 nm and 527 nm was observed in model-5. In PARAFAC model-6 (Fig. 4h and i), the first component ($\lambda_{\text{ex/em}}$: 295/300 nm with shoulder at 333 nm) detected in the models 4 and 5 was divided into two different components: yellow ($\lambda_{\text{ex/em}}$: 295/300 nm) and blue components ($\lambda_{\text{ex/em}}$: 295/330 nm), the latest representing the shoulder associated with the first component of models 4 and 5.

PARAFAC model-5 was optimised and validated after increasing the convergence criteria to 10^{-10} . PARAFAC models with five components were also validated for each species, and the peaks position was confirmed on the intraspecies datasets, although slight differences were observed on the excitation maximum of components one ($\lambda_{\text{ex}} = 275$ nm), four ($\lambda_{\text{ex}} = 385$ nm), five ($\lambda_{\text{ex}} = 465$ nm) for *Gryllus assimilis*, one ($\lambda_{\text{ex}} = 280$ nm), three ($\lambda_{\text{ex}} = 345$ nm), five ($\lambda_{\text{ex}} = 470$ nm) for *Gryllus bimaculatus* and on the emission maximum of several components among the considered species (Table 3).

3.3. Peak identification

Emission spectra recorded at the maximum of the absorption band (maximum excitation wavelength) of the analysed standard substances are displayed in Fig. 4. Several pure compounds and mixtures showed fluorescence peaks at excitation wavelengths ranging between 280 and 295 nm (tyrosine, tryptophan, tocopherol, albumin and mixtures of: tryptophan + tocopherol, tyrosine + tocopherol, tryptophan + tyrosine + tocopherol) and might be therefore suitable for explaining the first

two PARAFAC components. Similarly, PARAFAC component-3 ($\lambda_{\text{ex}} = 340$ nm) might be associated to pyridoxine, NADH, pterins and collagen. PARAFAC model-5 run on the overall averaged dataset was significantly improved when pure spectra of these compounds were added (Fig. 5).

Comparison between PARAFAC components and pure standard spectra showed good correspondence between component-1 and mixture of tocopherol, tryptophan and tyrosine (Fig. 6a), component-2 and albumin (Fig. 6b), component-3 and collagen, pyridoxine and pterins (Fig. 6c).

4. Discussion

To the best of our knowledge, this study represents the first investigation of the fluorescence landscape of edible insects. Although insect fluorescence has been largely explored, most of the studies investigated live insects and fluorescence phenomena connected with pigments responsible for their colourful teguments (Lagorio et al., 2015; Welch et al., 2012).

In the present work, EEMs of powders from 5 different edible insect species and 3 different origins were recorded and characterised. High complexity of their fluorescence landscape was observed, suggesting that edible insects are chemically complex matrices. Indeed, PARAFAC analysis conducted on the overall averaged dataset was validated for five components, indicating that five different peaks could be detected on the analysed EEM. This is in line with other traditional food such as meat, cheese and fish, where 4 or 5 fluorescence peaks are usually described (Andersen and Mortensen, 2008; Christensen et al., 2006; Islam et al., 2020).

Additionally, the five picked PARAFAC components were also detected on the intraspecies datasets, implying that fluorescence of several Orthoptera species raised from the same compounds. However, slight differences between samples could still be detected. Such

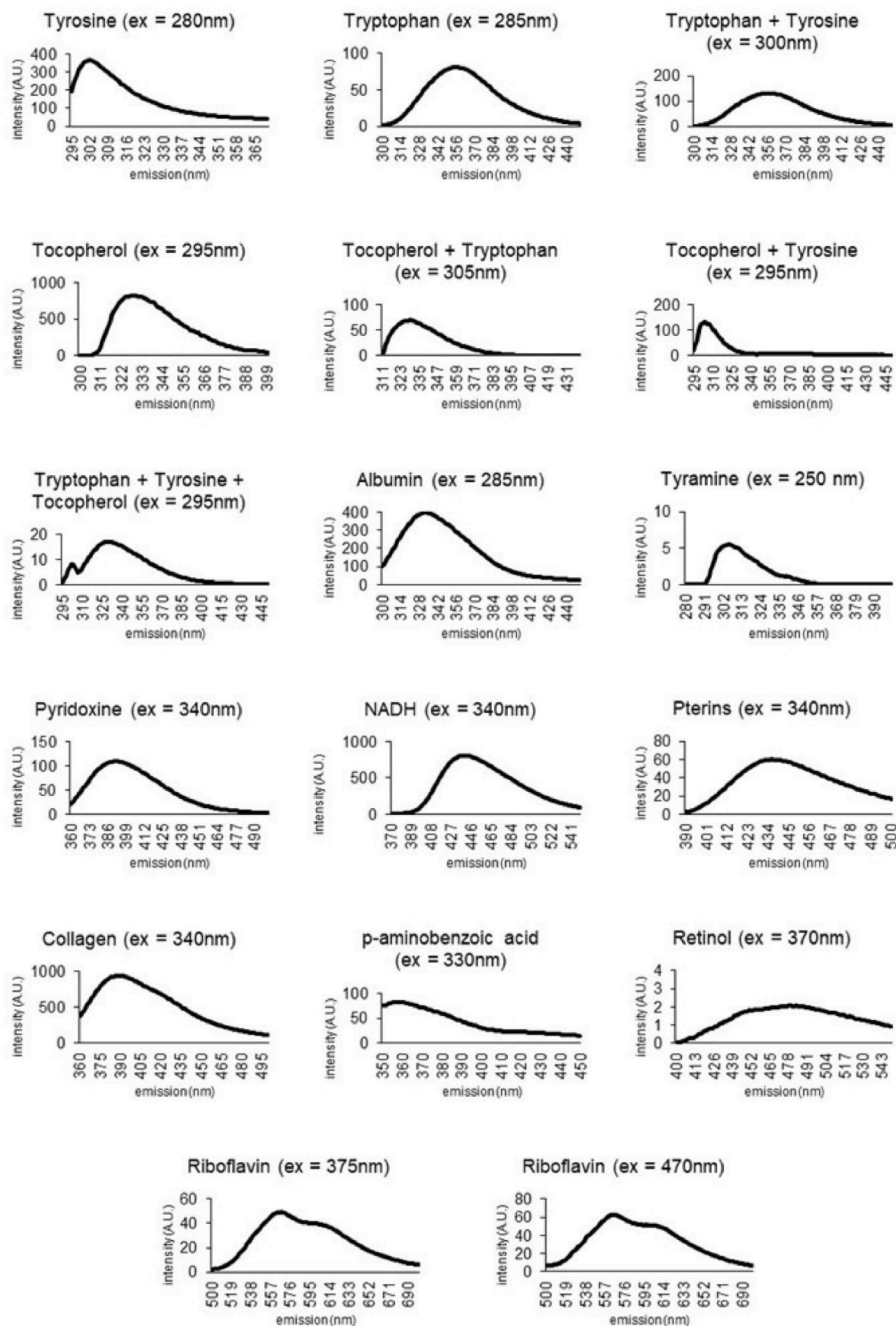


Fig. 4. Emission spectra of the standard pure compounds and mixtures recorded at their maximum absorption band (ex).

Table 3

Excitation and Emission maxima of the validated PARAFAC model on the overall averaged dataset and intraspecies datasets.

Dataset	PARAFAC model (Comp.)	Comp. 1 ($\lambda_{ex/em}$; nm)	Comp. 2 ($\lambda_{ex/em}$; nm)	Comp. 3 ($\lambda_{ex/em}$; nm)	Comp. 4 ($\lambda_{ex/em}$; nm)	Comp. 5 ($\lambda_{ex/em}$; nm)
overall	5	295/300; 333	285/333	340/392; 420	390/450	465/504-527
<i>A. domesticus</i>	5	295/299; 331	285/333	340/389; 425	390/452–457; 481	470/484-527
<i>G. assimilis</i>	5	295/302; 333	275/333	340/385; 422	385/443	465/484-527
<i>G. bimaculatus</i>	5	295/302; 335	280/334	345/392; 425-430	400/458	470/484-527
<i>L. migratoria</i>	5	295/299; 331	285/332	340/392; 425	390/457-481	470/504-540
<i>S. gregaria</i>	5	295/299; 340	285/332	340/391; 420	390/444-453	470/514-540

differences were mainly due to the position of the emission maxima of each PARAFAC component, which may be attributed to different physicochemical properties of the analysed powders. Indeed, it is well known that optical properties of each fluorophore depend on the environment where the specific fluorophore is located (Christensen et al., 2006). Furthermore, since several molecules may interact with the fluorophores (Sikorska et al., 2019), modifying their spectra, different chemical compositions of the analysed powders may lead to important differences in the peak position. This is relevant when different feeds are used for insect rearing as the diet has a strong impact on insect nutritional quality (Ojha et al., 2021; Van Huis et al., 2013). Therefore, if different feeds are provided to different insect species, fluorescence characterization and discrimination of insects as function of species and origin may be performed, as already shown on traditional products (Genis et al., 2019; Hammami et al., 2013; Lenhardt et al., 2017). However, since limited dataset was used in this experiment and the discrimination was not the object of this study, further and more focused research is needed in order to shed light on this aspect. Further differences between species and origins could also be highlighted on magnitude of the fluorescence peaks, which may be connected with different concentrations of the fluorophores in different samples. Indeed several studies have displayed that the PARAFAC loadings computed on sample mode pinpoint the relative concentration of the underlined fluorophore in each sample, allowing to use fluorescence for quantitative analysis (Bro, 1997; Sikorska et al., 2019). However, in order to implement fluorescence spectroscopy for chemical compounds quantification, characterisation of each fluorescence peak should be performed.

In the present study, identification of the compounds responsible for the fluorescence peaks was based on the idea that each PARAFAC component is associated with a specific chemical compound (Christensen et al., 2006; Murphy et al., 2013). Nevertheless, due to the high chemical complexity of edible insects (Rumpold and Schlüter, 2013), interaction between molecules can be expected, implicating that more compounds describe the same fluorescence peak.

Potential fluorophores responsible for edible insect fluorescence might be amino acids, vitamins, polyphenols, pigments and nucleic acid, which are the traditional fluorophores detected in food (Fernández-Romero and Aguilar-Caballos, 2019). Additionally, further compounds, such as purine and uric acid have been detected in some insect species (Melber and Schmidt, 1992; Mello and Vidal, 1985), therefore, they should be considered. In this study, the first two PARAFAC components exhibit excitation maximum at 285 and 295 nm, respectively. Proteins are usually excited in this region (Lakowicz, 2013). Edible insects are known to be a very important protein source, having concentrations ranging between 25 and 75% (Oonincx and Finke, 2020; Rumpold and Schlüter, 2013); therefore, it is possible to hypothesize that they are responsible for at least one fluorescence peak. Protein fluorescence origins from three aromatic amino acids, i.e. phenylalanine ($\lambda_{ex/em}$ = 260/282 nm), tyrosine ($\lambda_{ex/em}$ = 275/303 nm) and tryptophan ($\lambda_{ex/em}$ = 280/350 nm) (Lakowicz, 2013). Since proteins are usually excited at 280 nm, phenylalanine is not detected, while tryptophan and tyrosine could be detected in both, pure and combined forms. Therefore, PARAFAC component-2 (λ_{ex} = 285 nm) may be due to proteins. Specifically, perfect overlapping between this component and albumin spectra was observed, therefore component-2 might be associated to albumin.

However, since Montowska et al. (2019), studying the protein profile of cricket powders, did not report this specific protein, a mixture of several amino acids (i.e. tryptophan and tyrosine) may be a more suitable explanation for this peak. Indeed, tryptophan residues are usually located in the hydrophobic area of the proteins (Bussler et al., 2015) and such environment leads to a shift of their emission maximum from 350 to 320–330 nm (Albani, 2012), which is suitable for explaining the aforementioned PARAFAC component. Moreover, fluorescence peaks centred between 330 and 340 nm, detected in several other foods, such as fish and meat, are usually associated with this amino acid (Christensen et al., 2006). Nevertheless, since energy transfer from tyrosine to tryptophan is a common phenomenon happening within the proteins (Lakowicz, 2013), the aforementioned peak might also be the result of a mixture of tyrosine and tryptophan in hydrophobic environment.

Due to similar physical phenomenon, the PARAFAC component-1 (λ_{ex} = 295 nm) might also be associated with a mixture of tyrosine and tryptophan. However, since tyrosine cannot be excited at wavelengths higher than 290 nm (Lakowicz, 2013), it seems improbable that tyrosine is responsible for this fluorescence peak. Nevertheless, the detection of tyrosine at this wavelength cannot be excluded as an excitation slit width of 10 nm was used in this study, meaning that the excitation could fluctuate between 285 and 305 nm. Alternative explanation for the main component peak might be the Rayleigh or Raman scattering. A Raman effect was not observed due to the nature of samples (powder form) and it is typically due to absorption of solvents (Andrade-Eiroa et al., 2013). On the other hand, Rayleigh signal could be detected, even though preliminary correction was performed. Nevertheless, it might be excluded as it is usually beyond the measurement range of the instrument, while the studied peak is well defined. Therefore, the peak at emission 300 nm may be attributed to tyrosine. The shoulder at 333 nm is, instead, probably associated to tryptophan. However, excitation of 295 nm can also allow to detect tocopherol (i.e. vitamin E), which is an important fluorophore in several food products, such as milk, cheese, meat, oil, cereal flour and others (Altomonte et al., 2019; Christensen et al., 2006; Gherghina et al., 2021; Lenhardt et al., 2017). It shows a fluorescence peak centred at $\lambda_{ex/em}$ = 298/330 nm (Albani, 2012; Sikorska et al., 2019), therefore it can be expected to be co-responsible for this fluorescence peak.

PARAFAC component-3 was attributed to a mixture of pyridoxine, collagen and pterins. In particular, pterins, a class of chemical compounds responsible for pigmentation, nitrogen metabolisms, visual and neurohormonal activities (Ziegler and Harmsen, 1970), have been already detected in insects when emission spectra ranging between 420 and 455 nm over excitation 340 nm was considered (Abels and Ludescher, 2003; Bridges and Sohal, 1980; Kumazawa and Tabata, 2001; Melber and Schmidt, 1992). Pterins show a characteristic peak at $\lambda_{ex/em}$ = 350/450 nm (Lagorio et al., 2015); therefore, they can explain the shoulder of this PARAFAC component. However, since the same excitation wavelength has been used to detect NADH ($\lambda_{em,max}$ = 395 and 465 nm (Hassoun and Karoui, 2015; Sikorska, 2019)) in several food products (Christensen et al., 2006; Hassoun et al., 2020; Hassoun and Karoui, 2015; Sikorska, 2019; Sikorska et al., 2019), further analyses are requested in order to have a secure identification of the underlined peak. Other fluorophores usually detected in animal-derived food and able to emit fluorescence in the same region ($\lambda_{ex/em}$ = 350/420 nm) are

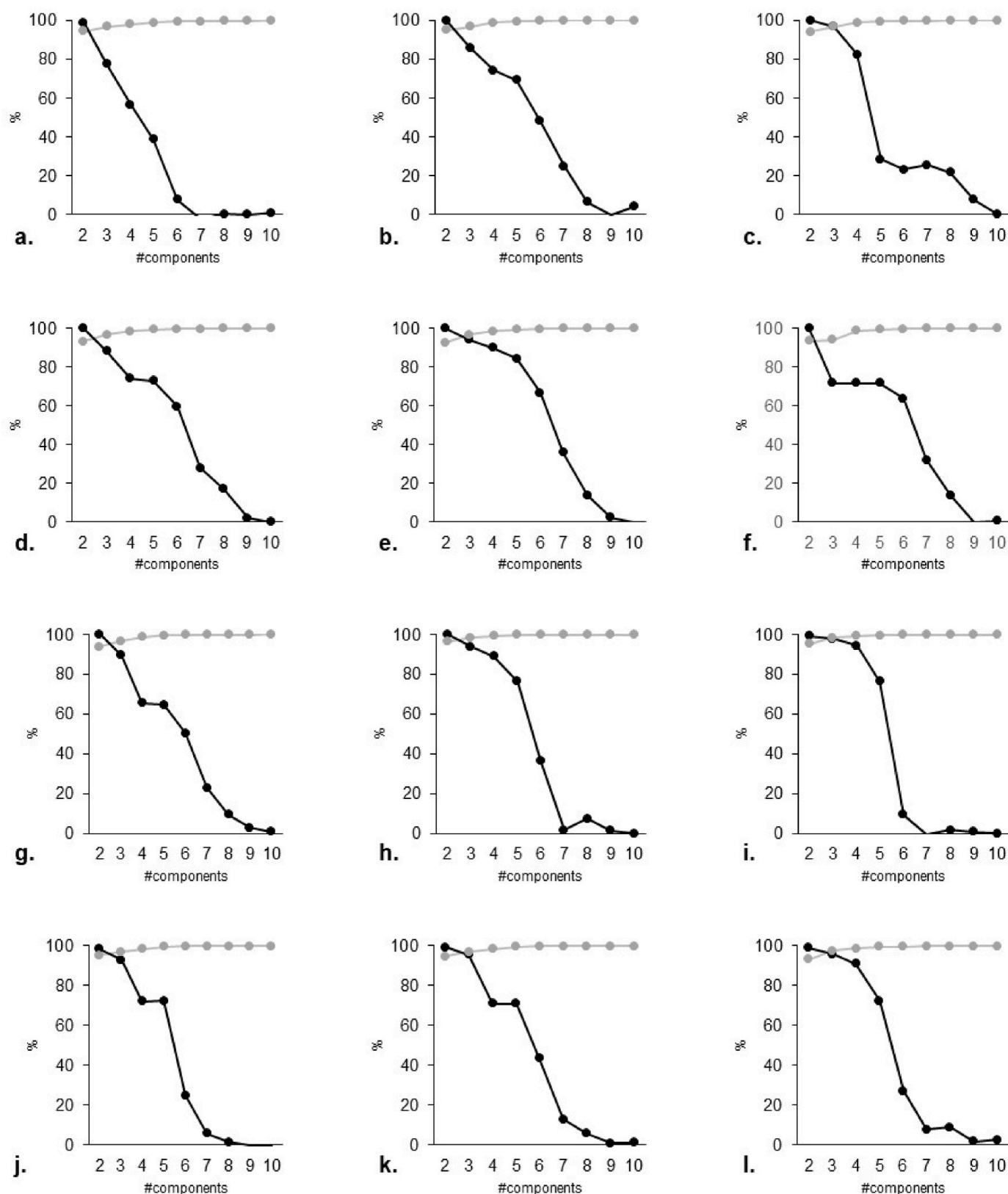


Fig. 5. Core consistency (black line) and percentage of explained variance (grey line) of PARAFAC analyses performed on the insect EEM after adding the EEM recorded from one single chemical standard compound (or mixture): tyrosine (a); tryptophan (b); tocopherol (c); tocopherol + tyrosine (d); tocopherol + tryptophan (e); tocopherol + tyrosine + tryptophan (f); tyrosine + tryptophan (g); albumin (h); collagen (i); NADH (j); pterins (k); pyridoxine (l).

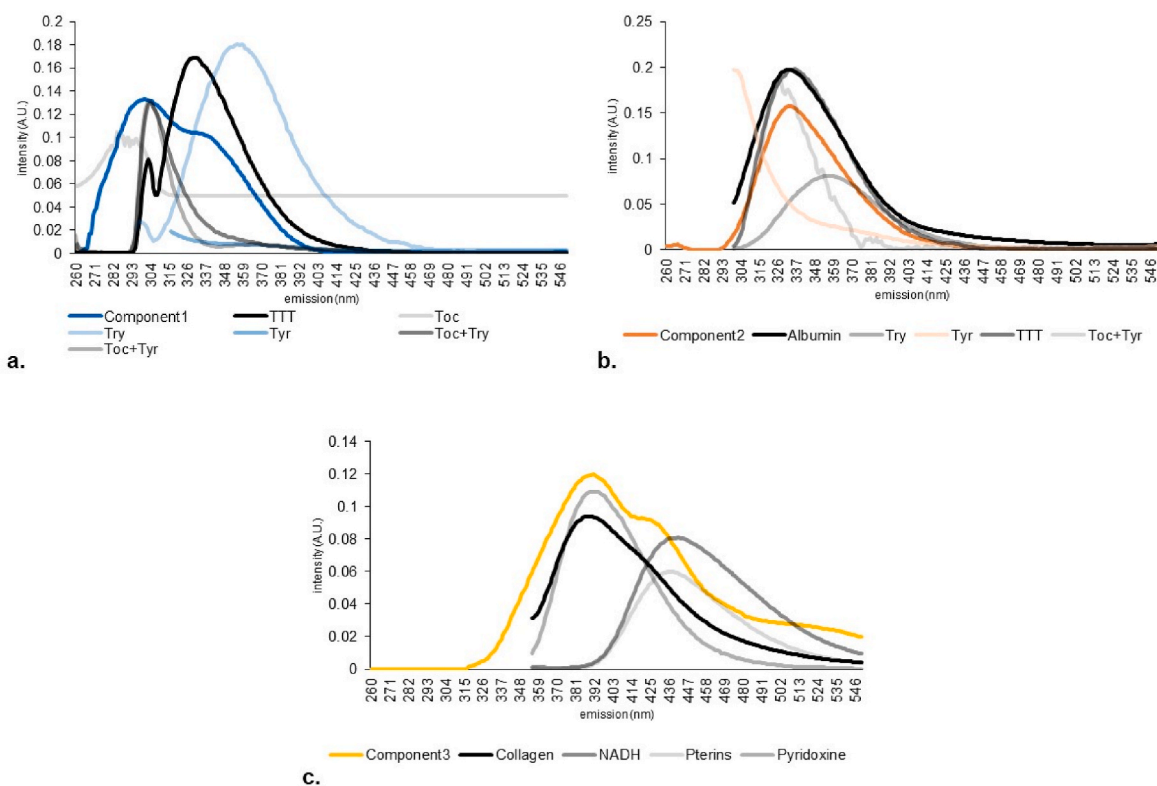


Fig. 6. Comparison between PARAFAC components 1, 2, 3 and spectra recorded from pure standard compounds. **a:** PARAFAC component-1, tocopherol + tyrosine + tryptophan (TTT), tocopherol (Toc), tryptophan (Try), tyrosine (Tyr), tocopherol + tryptophan (Toc + Try), tocopherol + tyrosine (Toc + Tyr). **b:** PARAFAC component-2, albumin, tryptophan (Try), tyrosine (Tyr), tocopherol + tyrosine + tryptophan (TTT), tocopherol + tyrosine (Toc + Tyr). **c:** PARAFAC component-3, collagen, NADH, pterins, pyridoxine.

represented by Schiff bases, originated through chemical reactions between saturated aldehydes and amino acids (Veberg et al., 2006). However, they are produced during the storage at high temperatures, and since the samples were stored for a short time at 4 °C, their presence can be excluded. Nevertheless, further analysis should be carried out in order to understand if such products can be used for monitoring storage time and conditions of edible insect powders. The main peak of the aforementioned PARAFAC component may instead be assigned to collagen ($\lambda_{ex/em} = 320/400$ nm (Skjervold et al., 2003)) and/or pyridoxine ($\lambda_{ex/em} = 340/400$ nm (Sikorska et al., 2004)). Pyridoxine (i.e. vitamin B6) is usually detected in high amounts in unprocessed insects (Ooninx and Finke, 2020) and displays strong fluorescence intensity, also if present in traces (Sikorska et al., 2004). Collagen is a structural protein, very common in animal tissue (Shoulders and Raines, 2009; Sutherland et al., 2013) and its fluorescence is often detected in meat (Christensen et al., 2006). Since the latter compound has been found in several insect species as well (Ashhurst, 1982; Ashhurst and Bailey, 1980; François, 1985), both pyridoxine and collagen are strong candidates for being described by the underlined PARAFAC component.

Due to the low signal/noise ratio recorded at excitation and emission higher than 400 and 450 nm, respectively, and to the high chemical complexity of this area, specific identification of the PARAFAC components 4 and 5 was not performed. However, since in the region where they were computed, vitamins (e.g. vitamin B2) and polyphenols (e.g. pterulic acid) (Ahmad et al., 2017; Christensen et al., 2005; Kahle et al., 2019; Yang et al., 2016) are usually detected, these peaks might be associated to mixtures of several vitamins and polyphenols. On the other hand, compounds potentially responsible for these two components may also be lipid oxidation products (Yamaki et al., 1992), already detected in meat and fish (Veberg et al., 2006). Therefore, in order to have an exact and punctual chemical identification of the latter two PARAFAC components, further studies should focus on the fluorescence in the

underlined region.

5. Conclusion

In the present study, the fluorescence landscapes of edible insects were investigated. PARAFAC analysis performed on EEM recorded from 15 insect powders was validated for five components suggesting that edible insect's optical fingerprint is characterised by five different peaks. The same PARAFAC components computed on the overall dataset were also detected in intraspecies dataset, pointing out that several insect powders have the same fluorophores. Slight differences in emission and excitation maxima, likely due to different feed and insect origin, could still be observed. Comparison between the PARAFAC components and emission spectra of standard compounds allowed to assign the first three components to mixture of tryptophan and tyrosine, mixture of tryptophan, tyrosine and tocopherol, mixture of pyridoxine, collagen and pterins, respectively.

In conclusion, this research shows that fluorescence spectroscopy can potentially be employed for fast monitoring of the composition of edible insects. However, further studies investigating other edible insect species and products thereof are needed in order to have a complete overview of the analytical potential of fluorescence in the edible insect sector.

Conflict of interest and Funding statements

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Microbial Growth and Physicochemical Quality Changes During Modified Atmosphere Storage of High Hydrostatic Pressure Processed *Tenebrio molitor* Paste

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Abstract

Fresh insects in the form of paste can be conveniently utilised for further food preparations and processing at both household and industrial levels. However, due to the high moisture content and particular physiochemical properties, insect paste is highly unstable and susceptible to significant quality changes during storage. The aim of this study was to evaluate the microbiological and physicochemical stability of high hydrostatic pressure (HHP) treated *Tenebrio molitor* paste stored at different modified atmosphere packaging (MAP) conditions. Three different MAP compositions and normal air were applied. pH, colour, lipid oxidation, fluorescence spectra and microbiological parameters were evaluated along 28 days of refrigerated storage (4 °C). HHP treatment (600 MPa, 5 min) resulted in a significant reduction in mesophilic aerobic bacteria, anaerobic bacteria and yeast and mould counts. Growth of mesophilic aerobic and anaerobic bacteria remained low for 7 days at 4 °C, regardless of the applied atmospheric conditions, while high microbial growth was observed after 14 days without any significant differences among the treatments. The number of mesophilic spores remained low throughout the entire storage period when MAP was applied, whereas high values were observed after 28 days in the samples stored in normal air. *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli* were not detected in any of the samples, nor in the raw paste. Lipid oxidation was initiated by the HHP treatment and displayed an increasing trend during the storage period, although the oxidation levels were lower in the MAP without oxygen compared to the normal air packaging and in MAPs with high O₂ or CO₂ levels. Fluorescence spectroscopy showed high potential for rapid detection of chemical changes in insect products during processing and storage. Overall, this study showed that MAP can effectively be applied on HHP-treated insect paste to extend its storage period. Absence of oxygen has been identified as a crucial factor in achieving reduced microbial growth and ensuring high physiochemical quality of the final product. Therefore, the present research offers a valuable insight for creating new edible insect-based products within the food industry.

Keywords Lipid oxidation · Edible insects · Fluorescence spectroscopy · Microbial inactivation · Shelf-life

Introduction

Edible insects are considered a high-value alternative protein resource for the future due to their high-quality nutrient compositions and low environmental impacts (Parodi et al., 2018).

The yellow mealworm (*Tenebrio molitor*) has recently been approved as a novel food by the European Commission, consequently allowing formulation of whole insects, insect parts and insect-based products including pasta, cookies and other food products (reg. EU 2022/169). However, fresh mealworms are usually contaminated with a diversity of microorganisms that may negatively affect safety and shelf-life of the final products (Osimani et al., 2018). Therefore, in order to ensure high food safety, insect processing and storage should follow similar health and hygiene standards as other traditional food (Ojha et al., 2021). Several decontamination methods including blanching, steaming, marination, drying, smoking, toasting and novel technologies have been proposed (Ojha et al., 2021). Additionally, processing of insects may result in powder or paste, which can allow the insects to be incorporated into food products or directly

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into dishes prepared by the consumer. While dry insect powder might be considered relatively stable during the storage, fresh insect paste, due to its high moisture content, favourable pH and rich nutrient profile, is highly perishable and prone to microbial and chemical changes upon storage (Rossi et al., 2023).

High hydrostatic pressure (HHP) processing is one of the most successful non-thermal processing technologies for decontamination of foods at an industrial scale. HHP can be applied to a range of raw and processed food products, usually in pre-packed forms. In recent years, HHP has also been tested for the decontamination of insect-based foods. For example, Kashiri et al. (2018) reported a reduction of more than 5-log in yeasts and mould counts in *Hermetia illucens* larvae after HHP treatment at 400 MPa for 2.5 min; however, no significant effect (only 0.35-log cycle reductions) was observed for total aerobic mesophilic microbial count. Additionally, Campbell et al. (2020) observed that HHP treatment at 400 MPa for 10 min applied to *H. illucens* larvae managed to reduce yeast and mould counts below the detection limits of 2-log cfu/g. Simultaneously, the treatment reduced Enterobacteriaceae counts (from 7.65 to 3.32 log cfu/g) and lactic acid bacteria counts (from 6.50 to 4.73 log cfu/g).

Modified atmosphere packaging (MAP) is a packaging system based on altering the composition of gases in contact with a food. It is widely used to extend the shelf-life and to improve the quality of fresh produce and perishable foods stored at refrigeration or freezing temperatures (Czerwiński et al., 2021). Nevertheless, MAP application on insect paste and other insect-based foods remains limited. One study observed slower microbial growth in mealworm paste packaged in 60% CO₂ and 40% N₂ compared to normal air (Stoops et al., 2017). On the other hand, De Smet et al. (2019) reported no microbial growth in mealworm paste stored at −21 °C rather than at 4 °C, where microbial growth was already detected during the first week.

To date, limited information is available regarding the long-term stability of HHP processed insect paste. For instance, there have been no investigations on using MAP in combination with HHP processing for insect-based products, despite its prior application in traditional foods such as meat and fish (Mao et al., 2023; Raso & Barbosa-Cánovas, 2003). Therefore, the aim of the present study was to investigate the effect of different MAP conditions on high-pressure-treated insect paste. Both microbial and physicochemical qualities were investigated during a 28-day period of refrigerated storage.

Materials and Methods

Sample Preparation and Treatment

Live mealworm larvae (*T. molitor*) were purchased from Fauna Topics GmbH (Marbach am Neckar, Germany).

Larvae were cleaned by sieving to remove frass and substrate impurities followed by packing in sterile bags and freezing at −30 °C until further use. Frozen larvae were blended into a paste with a knife mill (Grindomix GM200, Retsch GmbH, Haan, Germany) operating at 10,000 rpm, applying 2 cycles of 30 s. Mealworm paste were vacuum-sealed in polyethylene/polyamide bags and subjected to a HHP treatment at 600 MPa for 5 min. The treatment was carried out in a high-pressure single vessel apparatus U4000 (Unipress, Warsaw, Poland), operating at room temperature. Water solution of 50% isopropanol at an initial temperature of 20 °C was used as pressure medium. Pressure build-up took 1.22 min, during which the temperature rose to 38 °C, followed by quick decrease to 25 °C, and it remained constant until the end of the treatment. Pressure release was instantaneous.

Packaging, Gas Composition and Storage Parameters

HHP-treated mealworm paste samples (30 g; dry matter = 23.94 ± 0.23) were packed in 560-mL polypropylene trays (187 × 137 × 35 mm) and sealed on top with polypropylene film (thickness 30 µm; water vapour permeability at room temperature = 8 g/day·m²; DuPont Teijin Films US Limited, Richmond, VA, USA) using a packaging machine (Multivac T100, Germany). Packaging material was selected because of its low price, high heat resistant and low water permeability (Kumar et al., 2022; Mangaraj et al., 2009). Unconditioned air and three different modified atmospheres, viz., MAP2 (30% N₂, 70% CO₂, 0% O₂), MAP3 (60% N₂, 20% CO₂, 20% O₂) and MAP4 (70% N₂, 30% CO₂, 0% O₂), generated through a gas mixer (MAP Mix 9000, PBI Dansensor, Ringsted, Denmark) at a gas/product ratio of 15 mL/g were applied. These gas mixtures were selected based on gas compositions similar to that used in MAP of white meat products (Rossaint et al., 2014, 2015). Empty packages, i.e. without mealworm pastes, with respective gas mixtures were used for evaluating the net impact of the packaging material on the evolution of gas mixture composition within the package during storage. In total, 48 packages were prepared and were stored inside a walk-in, temperature-regulated room maintained at 4 °C for a period of 28 days. Storage time was determined based on the microbiology results demonstrated in previous studies by De Smet et al. (2019) and Stoops et al. (2017). These studies investigated the stability of pre-treated mealworm paste over 21 and 35 days of refrigerated storage, respectively. On day 0, 7, 14, 21 and 28, three packages each of treated samples were analysed for gas concentration inside the package as well as for physicochemical and microbiological quality parameters.

At each time point, O₂ and CO₂ measurements were performed before opening the package. The measurement was carried out using a portable gas analyser (Checkmate

3, PBI Dansensor, Ringsted, Denmark) extracting 2 mL of the air sample for each measurement.

Microbiological Analyses

The microbiological quality of the mealworm paste samples before and after HHP treatment and during cold storage was monitored assessing total aerobic mesophilic bacteria (AM), anaerobic mesophilic bacteria (ANM), aerobic mesophilic bacterial spores (MS) and yeast and mould (Y&M) counts. Briefly, 5 g of mealworm paste and 45 g of buffered peptone water were mixed in a sterile filter stomacher bag and homogenised (Bag Mixer Interscience, St. Nome, France) at speed 2 for 2 min. The homogenate was serially diluted with buffered peptone water in 1.5 mL sterile Eppendorf tubes. Plate count agar and Dichloran Bengal red chloramphenicol agar were used as medium for plating AM-ANM and Y&M, respectively. Microbial enumeration was carried out after 72 h of incubation at 30 °C for AM and ANM (DIN EN ISO 4833–2:2013) and after 5 days of incubation at 25 °C for Y&M (DIN EN ISO 13681:1995). Limiting for ANM, anaerobic conditions were generated by using anaerobic jar equipped with an anaerobic generation sachet (AnaeroGen, Thermo Fisher Scientific, Hennigsdorf, Germany) and an Oxoid Resazurin anaerobic indicator strip (Thermo Fisher Scientific, Hennigsdorf, Germany). For MS counts, two 1 mL aliquots of undiluted homogenate were boiled (80 °C, 20 min), cooled in ice and mixed together. Successive dilutions were carried out as for the other microbial groups, and nutrient agar with incubation at 37 °C for 48 h (DIN EN ISO 6887–1:1999) was used for enumeration. Detection limit of plate count analysis was defined at 200 cfu/g. All the analyses were conducted in duplicate.

Prevalence of *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* and *Clostridium perfringens* was studied on the raw, HHP-treated and 28 days stored products according to ISO 6579 (absence in 25 g), ISO AFNOR BRD 07/4–09/98 (absence in 25 g), ISO 16649–2:2001 and ISO 7937:2004, respectively.

pH and Colour Parameters

Colour measurements were recorded on the surface of the mealworm paste with a CFO200 colour sensor (Silicann Systems GmbH, Rostock, Germany) equipped with a two focus optics A2.0–100 scanner (Silicann Systems GmbH, Rostock, Germany). Working distance was set at approximately 95 mm, and 0° standard observer angle was applied. White reflectance standard WS-1-SL (Labsphere Inc., North Sutton, USA) was used as blank. Three different spots (Ø ~ 12 mm) were scanned for each sample, and

the recorded CIELAB colour space ($L^*a^*b^*$) values were mediated. Total colour difference was evaluated through the ΔE^* parameter, computed by applying the following equation (Serra-Castelló et al., 2023):

$$\Delta E^* = \sqrt{(L^* - L1^*)^2 + (a^* - a1^*)^2 + (b^* - b1^*)^2}$$

where L^* , a^* , b^* are the lightness, redness/greenness and yellowness/blueness, respectively, of the HHP-treated mealworm paste (time 0) and $L1^*$, $a1^*$, $b1^*$ are the CIELAB values measured on the paste stored for the respective time (7, 14, 21, 28 days).

pH was measured with a benchtop Lab 850 pH-metre (SI Analytics GmbH, Mainz, Germany) equipped with a WTW SenTix 41 electrode (Xylem Analytics GmbH, Weilheim, Germany). Approximately 1 g of mealworm paste was weighted in triplicate, dissolved in 10 mL of distilled water (1:10 v/v) and roughly homogenised, and the pH was recorded after 1 h of incubation at room temperature.

Lipid Oxidation

Detection and quantification of lipid oxidation were conducted applying TBARS method as described by Vyncke (1975) with slight modifications. Briefly, 2.5 g of mealworm paste were introduced in a 50-mL centrifugation tube and homogenised with 10 mg of butylated hydroxytoluene (Merck KGaA, Darmstadt, Germany; art. 128–37-0) and 10 mL of 5% (w/v) trichloroacetic acid (TCA; Merck KGaA, Darmstadt, Germany; art. 76–03-9) while keeping the samples in ice. Resulting material was centrifuged (10 min, 3900 rpm), and 3 mL of supernatant were added with 3 mL of 0.03 M thiobarbituric acid (TBA, Merck KGaA, Darmstadt, Germany; art. 504–17-6). Reaction was allowed at 100 °C for 1 h, and the absorbance of the final solution was read at $\lambda = 532$ nm, against a blank consisting in 3 mL of 0.03 M TBA dissolved in 3 mL of 5% (w/v) TCA. The following equation was applied for computing the malondialdehyde (MDA) concentration:

$$\frac{\text{mg MDA}}{\text{Kg sample}} = \frac{Y \times 0.44 \times 72}{\frac{1000}{\text{ml TCA}} \times \text{g sample}} \times 1000$$

where Y stands for the concentration (μM) of 1,1,3,3-tetramethoxypropane (TMP) obtained by replacing the absorbance at $\lambda = 532$ nm in the equation of a calibration line built by scanning several TMP solutions with different TBA concentration; 0.44 is the conversion factor accounting for the stoichiometry between MDA and TMP; 72 is the molecular mass of MDA; mL TCA indicates initial amount of TCA where the sample was extracted (10 mL) and g sample is the gram of initial sample (2.5 g).

Fluorescence Analysis

Fluorescence analysis was conducted in front face mode (incidence angle 60°) with a Perkin Elmer LS55 Luminescence spectrometer (Perkin-Elmer, Waltham, MA, USA) equipped with a pulsed xenon lamp and a red-sensitive photomultiplier (R928) detector. Emission spectra of the studied samples were collected at room temperature (20 ± 2 °C) at excitation range 250–295 nm in 5 nm steps and emission range 270–500 nm in 0.5 nm steps. Single scan mode was used, and Rayleigh scatter was manually deleted by keeping the minimum emission wavelength 20 nm higher than the excitation wavelength. Excitation and emission slit widths were both set at 2.5 nm, and data acquisition speed was 500 nm/min. In order to account for variation of the light source intensity, Raman peak was daily recorded in triplicate by scanning the emission spectra of Milli-Q water at excitation 350 nm and emission range between 370 and 500 nm.

Statistical Analysis

Mean centred and rhodamine corrected fluorescence data were normalised by dividing the recorded spectra for the Raman peak intensity, computed by integrating the daily averaged Raman spectra (3 scans/day) in the emission range 385–415 nm. Normalised spectra were reduced by deleting the emission wavelengths higher than 480 nm and organised in an excitation emission matrix (EEM) of dimension $54 \times 421 \times 10$ (number of samples $\times$ number of emission wavelengths $\times$ number of excitation wavelengths). In order to identify specific patterns in the data (i.e. number of peaks, position of peaks and peaks shift), EEM was submitted to Parallel Factor (PARAFAC) analysis, imposing non-negative constraints on all the modes (Andersen & Bro, 2003). Five PARAFAC models, from one to five components, were built by setting minimum convergence criteria of 10^{-6} , maximum number of interactions 2500 and repeating the fitting algorithm 10 times. Core consistency diagnostic, percentage of explained variance and split half analysis in 4 random splits were used to select and validate the right models (Stedmon & Bro, 2008). In order to evaluate possible hidden information in the fluorescence data, PARAFAC scores were plotted against TBARs data, and regression analysis was performed.

Microbiological and chemical data as well as colour parameters and maximum fluorescence intensity extracted from the highlighted spectra were submitted to one-way analysis of variance (ANOVA) with storage condition and time alternatively used as independent variable. Two samples *t*-test was used to compare treated and untreated samples at time zero. Shapiro–Wilk test and Levene’s test were used to verify normality and homoscedasticity of the model residuals, and whether linearity assumptions were

not respected, general linear model with gamma distribution (link inverse) was applied. Tukey’s HSD test at probably level of 0.05 was used for computing the differences among groups.

Raman normalisation, PARAFAC analysis, curve fitting and regression analysis were conducted in MATLAB R2021b (The MathWorks Inc., Natick, MA, USA), using drEEM toolbox version 0.6.3, while ANOVA, verification of linear model assumptions, data transformation and Tukey’s HSD test were carried out on R version 4.1.0 (R-CoreTeam, 2020), using “car” (Levene’s test), “bestNormalize” (arcsinh normalisation) and “agricolae” (Tukey’s HSD test) packages. Figures were generated in Microsoft Excel Professional Plus 2016 (Microsoft corporation).

Results and Discussion

Headspace Gas Composition, pH and Colour Measurements

Gas composition in the modified atmosphere package is extremely dynamic; therefore, it was monitored during the entire storage period. O₂ and CO₂ profiles in the different empty (control packages) and mealworm paste-filled packages are shown in Fig. 1. Increasing O₂ levels in MAP2 and MAP4 (both without oxygen) and decreasing CO₂ concentrations in MAP2, MAP3 and MAP4 were observed along the entire storage period. On the other hand, decreases of O₂ in MAP3 and air-packed paste and increases in CO₂ in air-packed samples were also detected. Such changes are common in MAP-stored animal products and are mainly due to packaging permeability, microbial growth and product activity, which may absorb CO₂ and O₂ resulting in oxidation (Esmer et al., 2011; Kirtil et al., 2016). Similar trends have also been reported in minced *T. molitor* and *Alphitobius diaperinus* food preparations stored 35 days at 4 °C under MAP (60% CO₂ and 40% N₂) or normal air (Stoops et al., 2017). However, in that study, headspace gas composition remained quite stable during the first 7 days of storage under MAP and 21 days of storage in normal air. The reason for these differences may be attributed to the product characteristics and packaging material. Specifically, Stoops et al. (2017) applied steaming and frying on mealworm paste, which allowed a higher microbial reduction than HHP. Moreover, the packaging used by Stoops et al. (2017), i.e. multilayers of polyamide, polyethylene and ethylene vinyl alcohol, was characterised by a very low gas permeability as shown by no composition change within empty packages. On the other hand, significant gas composition changes in empty packages, with increasing/stabilisation of O₂ to values close to 15–20% and decreasing of CO₂ to values close to

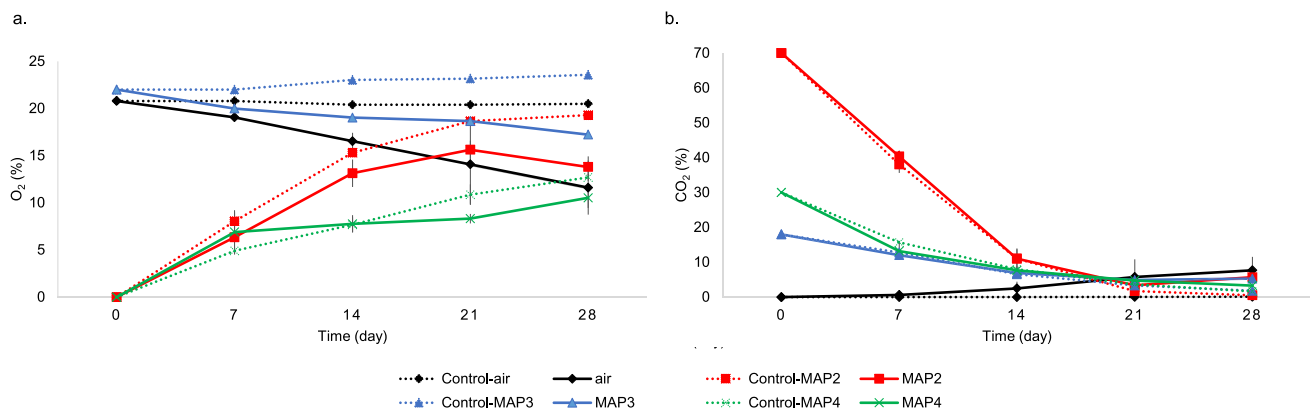


Fig. 1 Effect of modified atmosphere packaging (MAP) on the headspace oxygen (a) and carbon dioxide (b) concentration during storage (black, air; red, MAP2: 30% N₂, 70% CO₂, 0% O₂; blue, MAP3: 60% N₂, 20% CO₂, 20% O₂; green, MAP4: 70% N₂, 30% CO₂, 0%

O₂). Dash lines correspond to the control (packages without sample) of each treatment. Vertical bars correspond to the standard error computed on 3 replicates/treatment

0.1–1%, were observed in our experiment, where polypropylene containers with polypropylene lidding films were used. This can be attributed to higher O₂ (73×10^8 cm³ μm/(m² h atm)) and CO₂ permeability (88×10^7 cm³ μm/(m² h atm)) of polypropylene films than the materials used in the multilayer, such as polyamides (O₂ and CO₂ permeability of polyamide nylon is reportedly 31×10^8 and 39×10^7 cm³ μm/(m² h atm), respectively (Mangaraj et al., 2009)). Interestingly, headspace gas composition of both empty and paste-filled packages followed a fairly similar trend, especially in the packages without oxygen, suggesting that packaging permeability played a critical role when MAP was applied.

Significant differences between the different storage conditions on pH of the mealworm paste were detected only on day 7, while higher stability was observed during the following storage period (Table 1). Strong acidification was only observed during the first 21 days, regardless by the packaging used, while pH recorded at day 28 was not statistically different from the values measured at day 21. Such strong and rapid acidification may suggest microbial growth mainly concentrated during the initial 2 weeks of refrigerated storage. Borremans et al. (2018) also observed similar acidification of mealworm paste kept for 14 days at 35 °C and attributed such pH reduction to the combined activity of lactic acid bacteria (LAB) and sulphite-reducing bacteria (SRB). Moreover, storage conditions used in the study were extremely favourable for LAB development as already observed in *T. molitor* pastes stored at 4 °C for 3 weeks (De Smet et al., 2019).

Colour represents one of the most important quality parameters since it can directly affect the consumers' willingness to purchase and consume a certain food. Insect-derived products are particularly prone to colour change due to the naturally contain browning enzymes, which may strongly

affect the product quality (Bußler et al., 2016; Tonnejck-Srpova et al., 2019). Therefore, inactivation of such enzymes is paramount for ensuring longer shelf-life. In the present study, HHP treatment showed acceptable ability in colour stabilisation, confirming the findings of Tonnejck-Srpova et al. (2019), who observed effectiveness of HHP at pressures higher than 400 MPa on polyphenol oxidase and tyrosinase inactivation. Only slight, albeit still significant, colour changes were observed during the entire storage period. Such changes mainly concerned pastes stored in high oxygen atmosphere, i.e. air and MAP3 samples (Table 1) as showed by the ΔE parameter, which was significantly higher for these two treatments than for the others after 7 days of refrigerated storage. On the other hand, lower colour change was computed for MAP2 and MAP4 (i.e. the oxygen-free treatments), with ΔE similar to the other treatments only after 21 days. The observed differences could be explained by the higher oxygen concentration recorded in the headspace of the air and MAP3 packed pastes, which may have facilitated oxidation and therefore colour change (Kamau et al., 2018). Similar results were also reported by Tonnejck-Srpova et al. (2019), who displayed high colour stability when mealworm larvae were air-tight packed. Although progressive darkening (reduction of L*), accompanied by yellowness and redness reduction (reduction of b* and a*, respectively), has been observed, no clear over-time trend could be designed for L* and b*. It might be due to regional chromatic differences on the product surface, as already observed in meat (Esmer et al., 2011), as well as to syneresis and protein denaturation which may irregularly occur during the storage period (Tonnejck-Srpova et al., 2019). A clear decreasing trend was instead observed for parameter a*. Although this result was unexpected and it has never been reported in *T. molitor* paste, similar redness reduction was recorded in HHP-treated

Table 1 Mean $\pm$ standard deviation ($n=3$) of pH, L*, a*, b* and ΔE^* values of high-pressure-treated mealworm paste samples packaged under different modified atmosphere packaging conditions during the storage period

	Storage time (day)				
	0	7	14	21	28
pH					
Air	6.86 $\pm$ 0.02 ^A	6.48 $\pm$ 0.00 ^{bB}	6.09 $\pm$ 0.20 ^{aC}	5.86 $\pm$ 0.30 ^{aD}	5.75 $\pm$ 0.54 ^{aD}
MAP2	6.86 $\pm$ 0.02 ^A	6.61 $\pm$ 0.02 ^{aB}	5.97 $\pm$ 0.60 ^{aC}	5.66 $\pm$ 0.19 ^{aD}	5.68 $\pm$ 0.12 ^{aD}
MAP3	6.86 $\pm$ 0.02 ^A	6.56 $\pm$ 0.04 ^{aA}	6.18 $\pm$ 0.04 ^{aB}	5.77 $\pm$ 0.25 ^{aC}	5.70 $\pm$ 0.09 ^{aC}
MAP4	6.86 $\pm$ 0.02 ^A	6.62 $\pm$ 0.01 ^{aB}	6.16 $\pm$ 0.19 ^{aC}	5.72 $\pm$ 0.43 ^{aD}	5.31 $\pm$ 0.22 ^{aE}
L*					
Air	51.30 $\pm$ 2.24 ^A	39.75 $\pm$ 0.33 ^{abBC}	41.98 $\pm$ 0.06 ^{abB}	35.86 $\pm$ 0.37 ^{aC}	54.57 $\pm$ 0.29 ^{aA}
MAP2	51.30 $\pm$ 2.24 ^A	44.03 $\pm$ 0.38 ^{aB}	47.27 $\pm$ 0.09 ^{aAB}	38.02 $\pm$ 0.39 ^{aC}	52.57 $\pm$ 0.13 ^{aA}
MAP3	51.30 $\pm$ 2.24 ^A	35.80 $\pm$ 0.85 ^{bB}	36.31 $\pm$ 0.28 ^{bB}	36.71 $\pm$ 0.27 ^{aB}	39.15 $\pm$ 0.62 ^{cB}
MAP4	51.30 $\pm$ 2.24 ^A	42.80 $\pm$ 0.12 ^{aBC}	46.34 $\pm$ 0.25 ^{aB}	39.80 $\pm$ 0.35 ^{aC}	43.53 $\pm$ 0.06 ^{bBC}
a*					
Air	4.25 $\pm$ 0.45 ^A	2.22 $\pm$ 0.33 ^{bB}	1.77 $\pm$ 0.06 ^{bcB}	−2.09 $\pm$ 0.37 ^{bC}	−3.05 $\pm$ 0.29 ^{cD}
MAP2	4.25 $\pm$ 0.45 ^A	4.22 $\pm$ 0.38 ^{aA}	2.25 $\pm$ 0.09 ^{abB}	−0.91 $\pm$ 0.39 ^{aC}	−2.08 $\pm$ 0.13 ^{bD}
MAP3	4.25 $\pm$ 0.45 ^A	3.69 $\pm$ 0.85 ^{aA}	1.73 $\pm$ 0.28 ^{cB}	2.10 $\pm$ 0.27 ^{bC}	−2.31 $\pm$ 0.62 ^{bcC}
MAP4	4.25 $\pm$ 0.45 ^A	4.12 $\pm$ 0.12 ^{aA}	2.33 $\pm$ 0.25 ^{aB}	−1.51 $\pm$ 0.35 ^{abC}	−1.02 $\pm$ 0.06 ^{aC}
b*					
Air	9.93 $\pm$ 1.51 ^A	3.38 $\pm$ 1.30 ^{cBC}	2.14 $\pm$ 3.20 ^{bC}	7.72 $\pm$ 0.70 ^{bAB}	2.16 $\pm$ 0.40 ^{bC}
MAP2	9.93 $\pm$ 1.51 ^B	12.81 $\pm$ 0.80 ^{aA}	5.91 $\pm$ 0.30 ^{aC}	11.80 $\pm$ 1.54 ^{aAB}	5.58 $\pm$ 0.31 ^{aC}
MAP3	9.93 $\pm$ 1.51 ^A	7.40 $\pm$ 2.95 ^{bcA}	2.40 $\pm$ 0.17 ^{bB}	7.72 $\pm$ 0.63 ^{bA}	5.66 $\pm$ 1.36 ^{aAB}
MAP4	9.93 $\pm$ 1.51 ^A	11.25 $\pm$ 0.71 ^{abA}	5.41 $\pm$ 0.38 ^{aB}	11.18 $\pm$ 0.80 ^{aA}	6.75 $\pm$ 0.36 ^{aB}
ΔE^*					
Air	—	13.66 $\pm$ 3.02 ^{abA}	12.66 $\pm$ 2.79 ^{aA}	16.85 $\pm$ 1.49 ^{aA}	11.32 $\pm$ 1.94 ^{abA}
MAP2	—	7.88 $\pm$ 0.17 ^{bB}	6.69 $\pm$ 2.23 ^{bB}	14.39 $\pm$ 1.57 ^{aA}	7.94 $\pm$ 1.60 ^{bB}
MAP3	—	15.76 $\pm$ 3.10 ^{aA}	17.02 $\pm$ 1.69 ^{aA}	16.15 $\pm$ 0.18 ^{aA}	14.60 $\pm$ 1.88 ^{aA}
MAP4	—	8.62 $\pm$ 2.92 ^{bAB}	7.06 $\pm$ 1.25 ^{bB}	13.21 $\pm$ 2.81 ^{aA}	10.24 $\pm$ 1.05 ^{bAB}

Means with different capital letters in the same row are significantly different ($p \leq 0.05$)

Means with different lowercase letters in the same column in one category are significantly different ($p \leq 0.05$)

ground pork patties stored 9 days at 4 °C in the presence of oxygen and can be likely due to protein denaturation during the HHP treatment (Cunha et al., 2018).

Microbiological Quality

High water activity (estimated to be approximately 0.99 ± 0.01 (Borremans et al., 2020; Wynants et al., 2018)) and close to neutral pH make mealworm paste particularly prone to microbial contamination. Therefore, in order to guarantee proper food safety, appropriate processing technologies and storage strategies should be applied (Ojha et al., 2021). In this study, microbiological quality of mealworm paste was addressed across processing and storage by evaluating AM, ANM, MS and Y&M plate counts and the presence of *Salmonella* spp., *L. monocytogenes*, *E. coli* and *C. perfringens*. Results showed that untreated mealworm paste (raw) had high microbial counts (AM: 8.08; ANM: 7.99; MS: 3.19; Y&M: 4.48 log cfu/g, supplementary file 1). These high initial microbial counts could be explained

by the release of gut microbiota during the milling process (Ojha et al., 2021). For instance, Vandeweyer et al., (2017a, b) observed an increase of approximately 2-log cfu/g in the microbial load of *T. molitor* larvae after the milling process, which was considered as a suitable explanation of the higher initial microbial counts than other studies (Cacchiarelli et al., 2022; Mancini et al., 2019). However, the initial counts were consistent with most of the previous data published on mealworm paste (De Smet et al., 2019; Osimani et al., 2018; Stoops et al., 2016). In the current study, HHP at 600 MPa for 5 min resulted in approximately 2-log reduction in terms of AM and ANM (supplementary file 1). Similar results were also observed by Rumpold et al. (2014) with a reduction of 3-log after applying HHP at 600 MPa for 10 min on mealworm larvae. This stronger effect could be explained by the longer treatment time since different combinations pressure–temperature–time may result in different inactivation kinetics of vegetative microorganisms (Nema et al., 2022). No effect was observed on MS counts, confirming the limitation of HHP on bacterial spore inactivation (Nema

et al., 2022). On the other hand, a reduction of Y&M under the detection limit (200 cfu/g) was observed, in line with the findings of Larouche et al. (2019), who detected no yeast and mould residual on *Hermetia illucens* larvae after 3 min at 600 MPa.

Microbial growth dynamics during refrigerated storage are presented in Table 2. Vegetative bacterial counts increased during the time, exceeding the 7-log, which is considered the general spoilage level for food, after 14 days of storage and reaching approximately 9-log after 21 days. No effect of the different packaging conditions was observed, although MAP4 and MAP2 resulted in apparent (but not significant) slower microbial growth. Similar microbial dynamics were observed on steamed mealworm paste and steamed-fried larvae along 3 weeks of refrigerated storage (De Smet et al., 2019; Stoops et al., 2017).

However, significantly lower microbial growth was noticed in Stoops et al. (2017) when MAP with 60% CO₂/40% N₂ was applied. The reason for such discrepancy might be attributed to the difference in packaging material as well as the product type. While pure mealworm paste was used in the present study, Stoops et al. (2017) prepared a mixture of minced mealworm and several other ingredients which may have exercised a bactericidal or bacteriostatic

effect. MS count went through a slight decrease during the storage, except for air-packed samples. Similar trend was also observed in mealworm larvae stored for 6 days at 4 °C and was explained by their germination (Vandeweyer et al., 2017a, b). However, while a continuous reduction in spore counts was observed during the first 21 days of storage, further increase was detected from day 21 and day 28, regardless by the storage condition. Interestingly, Y&M were always under the detection limit, except for air-packed samples which were characterised by fungal contamination at the last sampling point. It suggests that the presence of oxygen during 28 days of chilled storage may favour a slight mould growth. Furthermore, the presence of Y&M (< 1-log cfu/g) in MAP samples as well as in air samples after 7 days of refrigerated storage might be a result of some artificial contamination during the handling, as outlined by the high standard deviation. A further explanation for the high microbial counts recorded on the product might be the applied repackaging operation. Specifically, while HHP is generally applied on ready-to-sell food (i.e. on food already packed within the final marketable packaging) ensuring high microbial safety standards and minimising any possibility of microbial re-contamination, transfer of the HHP-treated product to a new packaging was carried out in the present

Table 2 Mean ± standard deviation ($n=3$) of aerobic mesophilic bacteria (AM), anaerobic mesophilic bacteria (ANM), aerobic mesophilic bacterial spores (MS) and yeast and mould (Y&M) plate counts of high-pressure-treated mealworm paste packaged under different modified atmosphere packaging conditions during the storage period

	Time (day)				
	0	7	14	21	28
AM					
Air	5.94 ± 0.06 ^C	5.67 ± 0.12 ^{aC}	8.39 ± 0.17 ^{aB}	9.23 ± 0.28 ^{aA}	9.15 ± 0.15 ^{aA}
MAP2	5.94 ± 0.06 ^C	5.72 ± 0.07 ^{aC}	7.91 ± 0.43 ^{aB}	9.16 ± 0.18 ^{aA}	9.32 ± 0.03 ^{aA}
MAP3	5.94 ± 0.06 ^C	5.89 ± 0.13 ^{aC}	8.01 ± 0.14 ^{aB}	8.92 ± 0.33 ^{aA}	9.04 ± 0.27 ^{aA}
MAP4	5.94 ± 0.06 ^B	5.61 ± 0.19 ^{aB}	8.10 ± 0.15 ^{aA}	8.41 ± 0.87 ^{aA}	8.75 ± 0.52 ^{aA}
ANM					
Air	5.34 ± 0.00 ^C	5.50 ± 0.19 ^{aC}	8.34 ± 0.20 ^{aB}	9.26 ± 0.24 ^{aA}	9.15 ± 0.17 ^{aA}
MAP2	5.34 ± 0.00 ^C	5.48 ± 0.10 ^{aC}	7.74 ± 0.46 ^{aB}	9.08 ± 0.33 ^{aA}	9.32 ± 0.05 ^{aA}
MAP3	5.34 ± 0.00 ^C	5.74 ± 0.07 ^{aC}	8.01 ± 0.17 ^{aB}	8.92 ± 0.34 ^{aA}	9.01 ± 0.21 ^{aA}
MAP4	5.34 ± 0.00 ^B	5.37 ± 0.51 ^{aB}	8.11 ± 0.24 ^{aA}	8.57 ± 0.41 ^{aA}	9.00 ± 0.45 ^{aA}
MS					
Air	3.91 ± 0.13 ^B	2.17 ± 0.02 ^{aC}	1.55 ± 0.20 ^{bcD}	1.84 ± 0.21 ^{aCD}	4.45 ± 0.11 ^{aA}
MAP2	3.91 ± 0.13 ^A	2.29 ± 0.03 ^{aBC}	1.99 ± 0.27 ^{abC}	1.63 ± 0.29 ^{aC}	2.89 ± 1.78 ^{abB}
MAP3	3.91 ± 0.13 ^A	2.41 ± 0.38 ^{aB}	1.49 ± 0.10 ^{cC}	1.59 ± 0.19 ^{aC}	1.69 ± 0.38 ^{bC}
MAP4	3.91 ± 0.13 ^A	2.71 ± 0.23 ^{aB}	2.30 ± 0.15 ^{aBC}	1.75 ± 0.06 ^{aC}	2.11 ± 0.64 ^{abBC}
Y&M					
Air	n.d	0.87 ± 1.50	n.d	2.48 ± 0.26	2.59 ± 0.09
MAP2	n.d	n.d	n.d	n.d	n.d
MAP3	n.d	n.d	n.d	n.d	0.58 ± 1.00
MAP4	n.d	n.d	0.97 ± 1.69	n.d	0.80 ± 0.72

Means with different capital letters in the same row are significantly different ($p \leq 0.05$)

Means with different lowercase letters in the same column in one category are significantly different ($p \leq 0.05$)

n.d. not detected in 10⁻¹ dilution

study. This operation, although performed in a way that minimises possible interferences from the external environment, may have been responsible for further microbial contamination. Future studies should therefore investigate whether the absence of re-packaging can result in lower microbial re-contamination, shedding more light on the potentiality of the HHP-MAP combinations in industrial application.

Despite the high microbial counts recorded along the experiment, which may raise some concerns regarding the safety of the products, no pathogens as *Salmonella* spp., *L. monocytogenes*, *E. coli* and *C. perfringens* have been detected in such study, regardless of the applied packaging condition. Specifically, while *Salmonella* spp. and *L. monocytogenes* have never been detected on edible insects (Vandeweyer et al., 2021), *E. coli* and *C. perfringens* have been found in mealworm-derived products. As neither *E. coli* nor *C. perfringens* seem to be able of colonising live insect larvae (Cesaro et al., 2022; Van Looveren et al., 2022), their presence can be attributed to the cross-contamination during processing. Absence of these microbial species in the present experiment can conclude that sufficiently high hygienic standards were applied during the paste production procedure.

Lipid Oxidation

Lipid oxidation detected through the TBARs method showed an increasing trend during the storage, regardless of the packaging technology applied (Fig. 2). Such trend was clear during the first 14 days of refrigerated storage, while no statistically significant changes were observed at day 14, 21 and 28, within the same storage condition. The strong oxidation observed at the beginning of the experiment could be explained by the pre-treatment applied. HHP

is known for trigger oxidation reactions, not only favouring lipid globule breakage with increase of free fatty acids but also the cell membrane rupture with the release of metal ions and pro-oxidant enzymes which may initialise the oxidation process (Guyon et al., 2016; Medina-Meza et al., 2014). Pressures higher than 300–400 MPa are usually considered to be responsible for initialising such oxidation reactions, although different results have been observed in different products, with chicken meat usually more resistant than beef, pork, turkey and fish meat (Medina-Meza et al., 2014). Such difference has been attributed to the different fatty acid composition of these products, with unsaturated fatty acids (UFAs) more prone to oxidation than saturated fatty acids (Medina-Meza et al., 2014). Since *T. molitor* larvae are an important source of UFAs, and in particular oleic and linoleic acids (Rossi et al., 2022), it is reasonable to hypothesise that HHP treatment could have promoted the oxidation process. TBARs' value for HHP-treated samples was found to be significantly higher than untreated samples (supplementary file 1). Similar trend was also observed in black soldier fly larvae and oils extracted from mealworms and crickets (Larouche et al., 2019; Ugur et al., 2021). On the other hand, the relative stability in oxidative status observed at the end of storage period might be attributed to a significant reduction of enzymatic activity as well as to a reduction of free fatty acids or to a progressive degradation of the oxidation products (Guyon et al., 2016). Suitable explanation might also be found on the kinetic of the oxidative reaction, which may have reached an equilibrium point (Dominguez et al., 2019).

Concerning the different packaging conditions, significant differences ($p < 0.001$) between the different treatments have been observed along the entire storage period (Fig. 2). Samples packaged under normal air displayed the highest TBARs level, except after 21 days, where MAP3 paste showed significantly higher oxidation ($p < 0.001$). On the other hand, MAP4 offered the lowest oxidation level along the entire storage period. Interestingly, MAP2 and MAP4 (both without oxygen) showed similar behaviour during the first 21 days of storage, while MAP3 was significantly different from air packaged samples only at day 7. Such results suggest that MAP without oxygen might conveniently be used for limiting the oxidation process in HHP-treated mealworm pastes stored at refrigerated temperatures. Similar effects have also been observed in deep-fried crickets and ready-to-eat dry locusts, where absence of oxygen (obtained through vacuum packaging) was able to reduce lipid oxidation for at least 90 and 150 days of storage at room temperature, respectively (Gan et al., 2022; Ssepuuya et al., 2016). However, it should be noted that in both studies, no effect of vacuum packaging was observed whether antioxidant compounds or refrigerated storage was applied. On the other hand, significant reduction of lipid oxidation was observed

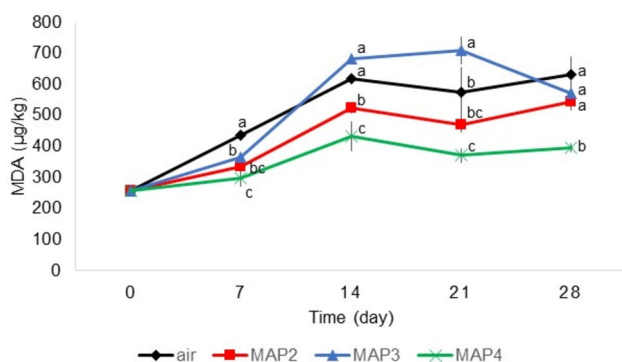


Fig. 2 Changes in malondialdehyde (MDA) concentration of high-pressure-processed mealworm paste stored at different modified atmosphere conditions (black, air; red, MAP2: 30% N₂, 70% CO₂, 0% O₂; blue, MAP3: 60% N₂, 20% CO₂, 20% O₂; green, MAP4: 70% N₂, 30% CO₂, 0% O₂). Vertical bars correspond to the standard error computed on 3 replicates/treatment; different lowercase letters at one specific storage day show significant differences ($p < 0.05$)

in vacuum packaged chicken meat kept 7 days in refrigerated conditions (Xiao et al., 2011), as well as in fish fillets stored 1 month at -3°C and in the absence of oxygen (Zhang et al., 2022). Thus, it is evident that the oxygen availability is a key aspect for inducing oxidation processes; therefore, limiting its presence can lead to higher lipid stability. Despite of it, in the present experiment, MAP2 showed lower performances than MAP4, with TBARs' level comparable to air and MAP3 pastes after 28 days of refrigerated storage. Suitable explanation for this might be related to the high gas permeability of the packaging used, with high oxygen concentration recorded in MAP2 packages ("Headspace Gas Composition, pH and Colour Measurements" section) after 7 days of storage.

Fluorescence Analysis

Fluorescence spectroscopy is a rapid and non-destructive analytic tool often used for monitoring quality and safety changes of food through processing and storage (Sikorska et al., 2019). Edible insect-derived products are characterised by several fluorophores (Rossi et al., 2021), which can be appropriately used for monitoring their quality. In the present study, a narrow region, ranged between excitation 250–295 nm and emission 270–500 nm, was considered. In such region, aromatic amino acids and tocopherol have been detected in several edible insect species (Rossi et al., 2021). Their fluorescence properties have been showed to be strictly associated to oxidation stability of several food, such as meat and other animal products (Domínguez et al., 2022; Loudiyi et al., 2022). PARAFAC analysis carried out on the recorded spectra suggested the presence of two well-defined components (supplementary file 2) which might be

associated to tryptophan but describe different physiochemical phenomena.

Specifically, PARAFAC component 1 ($\lambda_{\text{ex}}/\lambda_{\text{em}}=280/337.5\text{ nm}$) could mainly describe the red shift of the tryptophan spectra detected in samples submitted to HHP treatment (Fig. 3a). Shift of tryptophan emission spectra recorded at excitation 280 nm from lower ($\lambda_{\text{em}}=333.5\text{ nm}$) to higher ($\lambda_{\text{em}}=335.5\text{ nm}$) wavelengths has been already observed in several HHP-treated food, including *T. molitor* larvae and powder (Boukil et al., 2022; Bußler et al., 2016). It was mainly due to the protein unfolding which leads to tryptophan exposition to a polar environment, i.e. to the external surface of the protein (Andersen & Mortensen, 2008; Vivian & Callis, 2001). On the other hand, the second validated component ($\lambda_{\text{ex}}/\lambda_{\text{em}}=285/342\text{ nm}$) might be associated to the tryptophan concentration within the analysed samples. Reduction of maximum fluorescence peak recorded at excitation 285 nm was observed along the storage, irrespective by the applied condition (Fig. 3b). This phenomenon is extremely interesting and might be associated to an oxidative degradation of proteins as already described for other food products (Domínguez et al., 2022; Estévez et al., 2008; Sun et al., 2011; Utrera et al., 2012).

Several studies showed that protein oxidation could be kept low when packaging systems with low oxygen level were used (Hellwig, 2020). It was also confirmed in our study, where MAP conditions characterised by the absence of oxygen were effective for reducing the tryptophan degradation for at least 14 days of refrigerate storage. However, no statistically significant differences were observed between samples stored at different MAP conditions and normal air for more than 2 weeks ($F=2.224$, $p=0.163$).

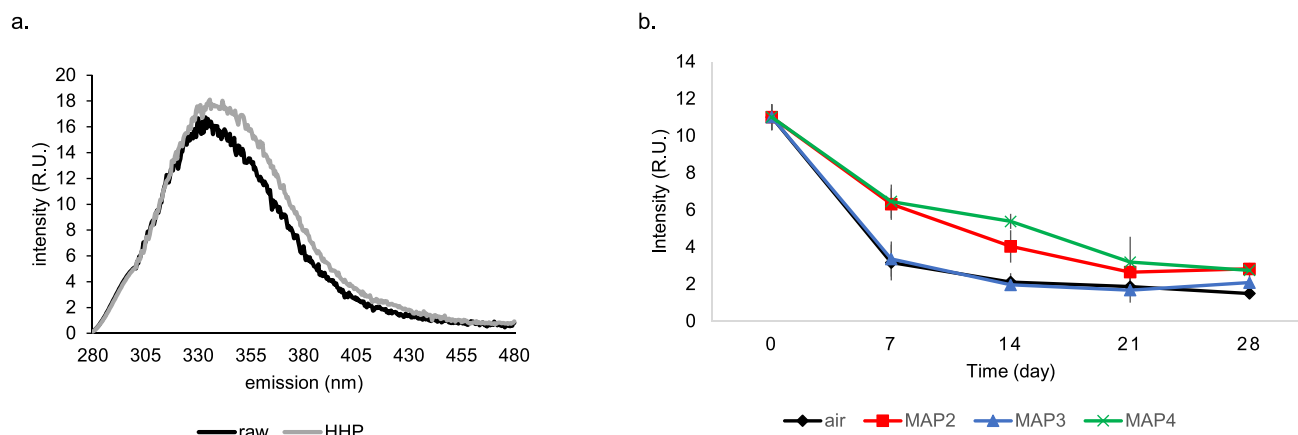


Fig. 3 Parallel Factor (PARAFAC) analysis of excitation emission matrix (EEM): **a** tryptophan emission spectra of raw and high hydrostatic pressure (HHP) processed mealworm paste samples, recorded at excitation 280 nm; **b** maximum fluorescence peak recorded at

excitation 285 nm (black, air; red, MAP2: 30% N_2 , 70% CO_2 , 0% O_2 ; blue, MAP3: 60% N_2 , 20% CO_2 , 20% O_2 ; green, MAP4: 70% N_2 , 30% CO_2 , 0% O_2). Vertical bars correspond to the standard error computed on 3 replicates/treatment

Regression analysis performed between PARAFAC component 3 scores (x) and TBARs (y) showed a significant (pseudo) first-order negative correlation ($y = 4661 \times^{-0.5522}$; $R^2 = 0.7802$; $p < 0.001$). It has already been observed in other studies (Domínguez et al., 2022; Estévez et al., 2008; Hassoun & Karoui, 2016) and further supports the theory that degradation of tryptophan fluorescence observed in our study is mainly due to oxidation process. However, additional analysis investigating a wider fluorescence region as well as using other more common techniques (i.e. protein carboxylation) should be performed in order to validate such statement.

Conclusions

HHP treatment (600 MPa, 5 min) resulted in a relevant reduction in aerobic mesophilic bacteria, anaerobic mesophilic bacteria and yeast and mould counts of mealworm paste; however, it did not have an effect on mesophilic spore count. Aerobic and anaerobic mesophilic bacteria counts increased during the refrigerated storage, exceeding the 7-log cfu/g, which is considered the general spoilage level for food, after 14 days of storage and reaching approximately 9-log cfu/g after 21 days, irrespectively by the MAP condition applied. Lipid oxidation detected through the TBARs method was promoted by HHP treatment and showed an increasing trend throughout the storage period. However, the oxidation process could be controlled by applying MAP without oxygen, and high quality of mealworm paste could be guaranteed for at least 14 days at 4 °C. Fluorescence spectroscopy showed potential for rapid detection of protein and lipid changes in insect products. In summary, the combined use of HHP and MAP could be an effective technology for maintaining the high physicochemical quality characteristics of insect paste during transportation, storage or retail display.

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Data Availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of Interest The authors declare no competing interests.

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Effect of high hydrostatic pressure processing on quality of antioxidant-enriched *Tenebrio molitor* paste

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Abstract

High hydrostatic pressure (HHP) is a non-thermal technology able to ensure microbial safety while preserving the natural properties of foods. However, several studies have shown high oxidation in HHP-processed food. Therefore, ability of antioxidants for controlling the oxidation of *Tenebrio molitor* paste was tested. Two commercially available antioxidant mixtures (Loxidan 303 and Loxidan E Ros Liquid +) were alternatively supplemented to fresh mealworm paste submitted to HHP treatment (600 MPa, 5min). Evolution of colour, pH, microbial status (total aerobic viable count, total anaerobic viable count, lactic acid bacteria), fluorescence intensity, lipid oxidation (acid value, peroxide value and TBARs) and fatty acid profile were investigated during 15 days of refrigerated storage. Results showed that HHP was able to significantly reduce microbial counts, browning index, acid value, peroxide value and TBARs. No lipid oxidation was observable during the treatment. Reduction in browning index and pH and increase in microbial counts were observed during the storage for all the pastes. Significant lower microbial contamination was detected after 15 days in treated paste containing Loxidan E Ros Liquid +. Peroxide values were constant in samples without antioxidants, while decreased in pastes containing Loxidan 303 and increased in pastes containing Loxidan E Ros Liquid + during the first 10 days of storage. Opposite trend was observed in the following 5 days. TBARs were stable in pastes without antioxidants, while increased in pastes with antioxidants. HHP appeared to be able on keeping their level low. Significant increase in PUFA for HHP treated samples was observed. Fluorescence analyses showed reversible unfolding of the proteins as well as high protein oxidation associated to HHP. Overall, the study suggested that HHP may extend the shelf-life of mealworm paste, with low lipid oxidation expected whether vacuum packaging was applied. In such conditions, antioxidant application should therefore be considered optional.

Keynotes edible insects, mealworm, HHP, antioxidant, oxidation

Highlights

- *Tenebrio molitor* paste were treated with HHP and stored for 15 days at 5°C
- HHP can reduce microbial contamination of *Tenebrio molitor* paste
- HHP does not trigger lipid oxidation in *Tenebrio molitor* paste
- Synthetic antioxidants were more active during and immediately after the treatment
- Natural antioxidants were more active during longer storage

1. Introduction

As consumers' interest toward fresh and minimally processed food is growing, novel non-thermal processing technologies are gaining attention (Kaur, Rambabu, Park, & Brett, 2022). Although traditional thermal methods display high performances for ensuring microbial safety, they can strongly impact nutritional, physical and organoleptic properties (Wang, Huang, Hsu, & Yang, 2016). High hydrostatic pressure (HHP) is a non-thermal technology that has shown promising results in microbial reduction, while preserving the original quality of food products (Aganovic et al., 2021; Kaur et al., 2022).

Effects of HHP on quality and safety have been studied on several products, including fruit and vegetables, juices, beer and wine, egg products, milk, seafood, meat, yogurt, jam and jellies (Wang et al., 2016). Application of HHP includes microbial reduction, enzymes activation/deactivation, protein denaturation, nutrient extraction, texture modification (Bermúdez-Aguirre & Barbosa-Cánovas, 2011). However, despite many positive effects, HHP has also shown some drawbacks, such as lipid and protein oxidation (Medina-Meza, Barnaba, & Barbosa-Canovas, 2014). Recent studies on chicken meat have displayed that HHP at 600 MPa induces the formation of secondary oxidation products (Alves, Bragagnolo, da Silva, Skibsted, & Orlén, 2012), while lipid oxidation was detected in frozen haddock and mackerel minces treated at 200 MPa, rather than at 300 MPa (Cropotova et al., 2020). Therefore, defining the right balance between treatment conditions is crucial for achieving the desired effects and minimizing disadvantages.

Edible insects are considered a novel food product that is attracting interest from a growing segment of the population (Boukid, Sogari, & Rosell, 2023). Due to their capacity to grow on by-products and waste while generating high quality biomass, consuming minimal resources and emitting low levels of greenhouse gasses, edible insects have been identified as a promising way to face the problems of food

security (Lumanlan, Williams, & Jayasena, 2022). The yellow mealworm, *Tenebrio molitor*, is considered one of the most promising edible insect species. However, while whole unprocessed or minimally processed insects might be appealing in countries with a strong tradition of eating insects, Western countries may still not be ready for embracing this food (Melgar-Lalanne, Hernández-Alvarez, & Salinas-Castro, 2019). Processing the insects into flours, pastes or nutrients to be integrated into traditional products may represent the key for promoting their widespread use. Recent studies have shown that *T. molitor* can be effectively treated by HHP, resulting in a microbiologically safe product, with improved functional properties and more suitable for inclusion in traditional formulations (Bolat, Ugur, Oztop, & Alpas, 2021). However, lipid oxidation, likely triggered by HHP, has been observed (Ojha et al., 2023). Therefore, the aim of this research was to investigate whether the addition of specific antioxidants to fresh mealworm paste could reduce the negative effects of HHP. Physical, chemical and microbiological parameters of treated and untreated pastes were therefore investigated over 15 days of refrigerated storage.

2. Materials and Methods

2.1 Raw material

Last stage mealworm larvae were provided by Fauna Topics GmbH (Marbach am Neckar, Germany). Upon their arrival, they were separated from the residual frass through sieving, starved for 1 day at 27°C and 70% RH, and inactivated by freezing at -30°C.

Two commercial antioxidant premixes, named Loxidan 303 and Loxidan E Ros Liquid + were provided by Kaesler Nutrition GmbH (Cuxhaven, Germany). The first mixture (303) was represented by a synthetic antioxidant containing 20% of Propyl gallate, 10% of Butylated hydroxyanisole (BHA) and 10% of Citric acid; the second mixture (E Ros Liquid +) consisted in a natural antioxidant containing tocopherol extracts (18.9%) and rosemary extracts (64.3%).

2.2 Experimental set-up

Frozen larvae were thawed overnight at room temperature (~20°C) and separated in three different batches. Loxidan 303 was added to the first batch (final concentration = 500 mg/kg), while Loxidan E Ros Liquid + was added to the second batch (final concentration = 1500 mg/kg). No antioxidant was

added to the third batch. Final concentrations of each antioxidant were defined based on the producer's suggestion.

After adding the antioxidants, each batch was separately milled into a paste by using a hand blender (MSM6M810, Robert Bosch GmbH, Germany). Generated pastes were separated in two further batches (figure 1) and packed under 94% of vacuum in PA/PE 20/70 cooking bags (beutel24.com, Wuppertal, Germany). Of the two batches, one was subjected to HHP treatment (samples T), while the other one was left untreated (samples U). HHP treatment was carried out in a high-pressure single vessel apparatus U4000 (Unipress, Warsaw, Poland) operating at room temperature (20-25°C). Pressure of 600 MPa was applied for 5 min. Treatment conditions were selected as they are the most common for industrial application (Aganovic et al., 2021). Pressure build-up time was 1.22 min, while release was instantaneous. Pressure transmitting medium consisted in a 50% isopropanol solution.

In order to gain an indication on the stability of the different mealworm pastes during the storage, three storage time (5-, 10-, 15- days) were considered, resulting in overall 24 different treatment/time combinations. Storage was carried out in a kitchen fridge set at 5°C and ensuring complete dark. These conditions were defined as considered to be reliable for insect pastes (Ojha et al., 2023).

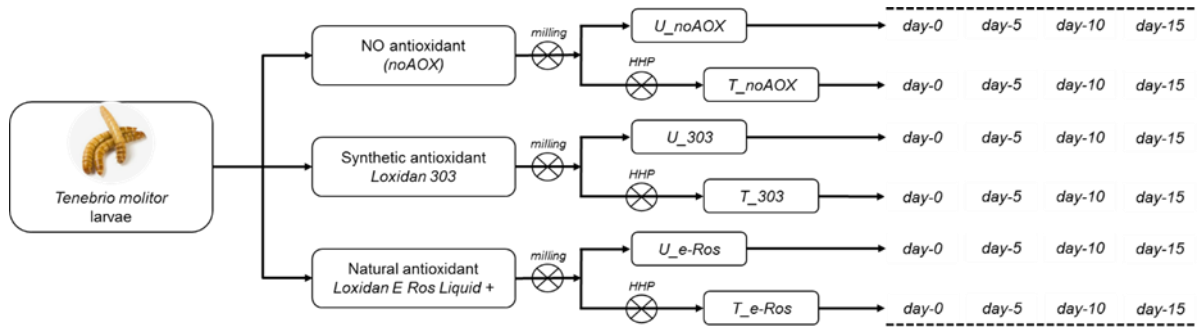


Figure 1. Graphical illustration of the experimental plan.

2.3 Proximate analysis

Proximate analysis of the mealworm pastes was only carried out on day-0 pastes by analysing dry matter, total ash, crude lipids, crude proteins, neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL). Analyses were performed according to Rossi, Ojha, Müller-Belecke, and Schlüter (2023).

2.4 Fatty acids composition

Fatty acid profile was analysed on day-0 and day-15 samples. Fat extraction was carried out according to the Soxhlet method (AOAC, 2002), avoiding the recovery step and evaporating the obtained lipids with a rotavapor R-215 (BÜCHI, Flawil, SG, Switzerland). Fatty acid profile was determined following the procedure for fatty acid transmethylation described in Caligiani et al. (2019) with some modifications: 50 mg of the Soxhlet extracted lipids were dissolved in 0.5 mL sulfuric acid:methanol (1:15 v/v) and heated for 3 h at 100°C. The resulting solution was added with 100 µL of internal tetracosane standard 1000 ppm and diluted to 5 mL with hexane. This solution was further diluted with hexane to match the linearity range of the instrument and injected into a Thermo Scientific Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with a Supelcowax MS capillary column (Sigma-Aldrich, Saint Louis, MO, USA) coupled with a Thermo Scientific Trace ISQ mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Helium was applied as carrier gas, and the Fatty Acid Methyl Esters (FAMES) standard mixture Supelco 37 (Sigma-Aldrich, Saint Louis, MO, USA) was the standard reference.

2.5 Antioxidant recovery

The concentration of the antioxidant compounds was measured in day-0 and day-15 pastes. Recovery of propyl-gallate and BHA was performed by extracting 5g of sample in 10 mL of 1:1 (v:v) methanol:acetonitrile solution under sonication. Clear solutions were directly injected into the mobile phase (50% mixture of acetonitrile-methanol moving at 0.8 mL/min) of a LC-20AT Shimadzu HPLC system (Shimadzu Deutschland, Berlin, Germany) equipped with a Shimadzu SPD-20 AV UV/Vis detector and a RP-C₁₈ Metacil (5 µm) ODS separation column.

Tocopherols were extracted by mixing 2 g of the sample with 20 mL of hexane under sonication. Clear solutions were injected in the same HPLC system as above, equipped with a FR-10 AXL fluorescence detector (Shimadzu Deutschland, Berlin, Germany) set at excitation/emission wavelengths of 290/325 nm, and a normal-phase Supelcosil LC-Diol separation column (Sigma-Aldrich, St. Louis, Missouri, USA).

2.6 Colour, pH and fluorescence measurements

Colour was recorded with a CFO200 colour sensor (Silicann System GmbH, Rostock, Germany) equipped with a two-focus optics A2.0-100 scanner (Ojha et al., 2023). For each paste, CIELAB colour

space ($L \cdot a \cdot b$) was measured on three different spots ($\varnothing \sim 12$ mm) by using a white reflectance standard WS-1-SL (Labsphere Inc., North Sutton, USA) as blank. Values recorded in each spot were mediated. Brown index (BI) was computed according to De Smet et al. (2019):

$$(eq. 1) \quad BI = 100 * \frac{x-0.31}{0.17}$$

Where x was determined by applying the following equation (eq. 2): $x = \frac{(a + 1.75L)}{5.645L + a - 3.012b}$

pH of fresh and stored mealworm pastes was measured with a benchtop Lab 850 pH-meter (SI Analytical Lab, Mainz, Germany), equipped with a WTW SenTix 41 electrode (Xylem Analytics GmbH, Weilheim, Germany) (Ojha et al., 2023).

Front face fluorescence spectra were recorded by scanning the pastes with a Perkin-Elmer LS55 Luminescence device (Perkin-Elmer, Waltham, MA, USA) equipped with a pulse xenon lamp and a red-sensitive photomultiplier (R928) detector. Incidence angle between sample and light beam and scanning speed were 60° and 500 nm/min, respectively, while excitation and emission slits were set at 2.5 nm. Emission spectra (0.5 nm steps) were recorded at 3 different excitation regions, encompassing the wavelengths 280-290 nm (region-1), 320-350 nm (region-2) and 380-390 nm (region-3), each one in 5 nm steps. Emission spectra of MilliQ water recorded at excitation of 350 nm was used as reference (Rossi, Durek, Ojha, & Schluter, 2021).

2.7 Fat oxidation

Acid value (AV) and peroxide value (POV) were determined on Folch extracted fat, while TBARs were directly measured on the pastes. Folch extraction was carried out according to Mopuri et al. (2021).

For AV, approximately 1 g of Folch extracted fat was re-solubilized in 10 mL of isopropanol:toluol (1:1) solution and titrated with 0.1 N KOH solution (Purschke, Stegmann, Schreiner, & Jäger, 2017).

AV (mg KOH/g fat) was determined according to equation 3:

$$(eq. 3) \quad AV = \frac{V1 * N1 * PM1}{W}$$

Where: $V1$, $N1$ and $PM1$ is the volume (mL), the normality (0.1 N) and the molecular weight (56.10 g/mol), respectively, of the KOH solution used for neutralizing the acidity of the initial fat solution, while W is the weight of the titrated fat (1 g).

POV (meq O₂/kg fat) was determined according to Lenaerts, Van der Borght, Callens, and Van Campenhout (2018) and computed according to equation 5:

$$\text{POV} = \left(\frac{(V_s - V_b) * N}{W} \right) * 1000$$

Where V_s is the volume (mL) of sodium thiosulfate used for neutralizing the fat solution, V_b is the volume (mL) of sodium thiosulfate used for titrating the blank, N is the normality (0.001 N) of sodium thiosulfate and W is the weight of the titrated fat.

TBARs (mg malondialdehyde/kg sample) were determined according to Ojha et al. (2023).

2.8 Microbial analysis

Total aerobic viable counts (TVC-aerobic), total anaerobic viable counts (TVC-anaerobic) and lactic acid bacteria (LAB) were determined along the storage. For each treatment/time combination, 5 g of paste were mixed with 45 mL of buffered peptone water and homogenised in a sterile stomacher bag at speed 2 for 2 min. Serial dilutions were performed in buffered peptone water and selected dilutions were plated in duplicate. Microbial enumeration was carried out after 72h of incubation at 30°C on Plate Count Agar (PCA) for TVC-aerobic and TVC-anaerobic (DIN EN ISO 4833-2:2013) or on De Man-Rogosa-Sharpe (MRS) agar for LAB (DIN EN ISO 10109:2016-05). Anaerobic conditions (for TVC-anaerobic) and microaerophilic conditions (for LAB) were generated within 2.5 L anaerobic jars by using AnaeroGen Oxoid Sachet (Thermo Fisher Scientific, Hennigsdorf, Germany) or CampyGen Oxoid Sachet (Thermo Fisher Scientific, Hennigsdorf, Germany), respectively.

2.9 Statistical analysis

Each analysis was carried out in duplicate and results were averaged. Three independent replicates for each treatment/time combination were analysed. Preliminary linear models were built as function of each variable and models residuals were submitted to Shapiro-Wilk and Levene's tests for verify normality and homoscedasticity assumptions. Analysis of the variance (ANOVA) or General Linear Model (GLM) with quasipoisson distribution (link "log") were applied. Probability level of 0.05 was considered, and Tukey HSD post-hoc test was applied when significant differences were detected. One-way ANOVA was used for the chemical proximate results as well as the fatty acid data. Two-sample t-test was applied for analysing the overtime variation of antioxidant recovery and fatty acids,

within the same sample. AV, POV, TBARs, BI, pH and microbial data were analysed through two-way ANOVA, with treatment and time as independent variables.

Principal Component Analysis (PCA) was performed on mean centred and normalised fatty acid data. PCA scores associated to the first two principal components (explaining more than 10% of the overall variance) were therefore submitted to analysis of similarities (ANOSIM) with 999 permutations (Anderson, 2001).

Three-dimensional fluorescence data were Raman normalised and resolved through the Parallel Factor (PARAFAC) analytic procedure. PARAFAC scores associated to each component were then submitted to two-way ANOVA.

Statistical analyses were conducted under R statistical environment (R-CoreTeam, 2020). PARAFAC analysis was carried out on MatLab 2017b, using the drEEM toolbox (Murphy, Stedmon, Graeber, & Bro, 2013), while ANOSIM was performed on PRIMER 7 (Anderson & Walsh, 2013).

3. Results and discussion

3.1 Chemical composition of insect paste

Chemical composition of *T. molitor* pastes is displayed in table 1. Although some differences were observed between the different treatments, proximate composition of mealworm pastes appeared stable suggesting that no important effect in terms of proximate composition can be associated to HHP treatment. Dry matter of the investigated pastes ranged between 23.76 and 24.90%, which laid within the published range (20-30%) (De Smet et al., 2019; Kröncke & Benning, 2023; Ojha et al., 2023). Similarly, protein level was stable and comparable to published data (50-60%) (Kröncke et al., 2019; Osimani et al., 2017; Ribeiro et al., 2024), while lipids were significantly lower than in previous studies (27-30%), which may be due to difference in diet and rearing conditions (Lenaerts et al., 2018; Ribeiro et al., 2024; Rossi et al., 2022). Further, a lower lipid content was measured in HHP treated pastes (10% compared to 12% in untreated pastes). This data is in line with Ugur, Bolat, Oztop, and Alpas (2021) and might be due to the formation of protrusions on the cells surface which being not completely collapsed may prevent the release of the cells content (Tuhanioglu, Alpas, & Cekmecelioglu, 2022), resulting in lower lipid recovery. Similar results have been observed in fish rest raw materials and lamb

meat treated with HHP at 400 and 600 MPa (Kotsoni et al., 2024; Q. L. Ma, Hamid, Oey, Kantono, & Farouk, 2020), while lower pressures have often shown enhanced fat extraction yields (Tuhanioglu et al., 2022).

Table 1. Chemical composition (% dry matter, average $\pm$ standard error) of the different mealworm paste used in the experiment. Value followed by different letters within the same row are statistically different (p-value < 0.05).

Parameter	U_noAOX	U_303	U_e-Ros	T_noAOX	T_303	T_e-Ros
Dry matter (%FM)	23.76 $\pm$ 0.11 e	23.83 $\pm$ 0.02 de	24.19 $\pm$ 0.10 bc	24.11 $\pm$ 0.03 cd	24.48 $\pm$ 0.06 b	24.90 $\pm$ 0.01 a
Ash	6.63 $\pm$ 0.07 b	6.64 $\pm$ 0.09 b	6.42 $\pm$ 0.02 b	8.48 $\pm$ 0.41 a	7.24 $\pm$ 0.18 b	6.81 $\pm$ 0.21 b
Protein	54.90 $\pm$ 0.59 ab	54.39 $\pm$ 0.24 ab	53.66 $\pm$ 0.05 b	55.38 $\pm$ 0.07 a	54.78 $\pm$ 0.11 ab	54.26 $\pm$ 0.04 ab
Lipids	12.76 $\pm$ 0.21 a	11.86 $\pm$ 0.79 ab	11.09 $\pm$ 0.10 ab	9.59 $\pm$ 0.28 c	9.96 $\pm$ 0.05 c	10.43 $\pm$ 0.30 bc
NDF	15.06 $\pm$ 0.32 a	15.55 $\pm$ 0.27 a	15.45 $\pm$ 0.08 a	14.25 $\pm$ 0.30 a	14.79 $\pm$ 0.17 a	15.07 $\pm$ 0.79 a
ADF	9.43 $\pm$ 0.13 a	9.61 $\pm$ 0.28 a	9.91 $\pm$ 0.19 a	9.29 $\pm$ 0.12 a	9.40 $\pm$ 0.17 a	8.30 $\pm$ 0.83 a
ADL	1.30 $\pm$ 0.08 a	1.34 $\pm$ 0.06 a	1.58 $\pm$ 0.11 a	1.28 $\pm$ 0.14 a	1.53 $\pm$ 0.09 a	1.02 $\pm$ 0.21 a

FM, fresh matter; NDF, neutron detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin

3.2 Effect of HHP on colour and pH

Physical properties (BI and pH) of mealworm pastes are displayed in table 2. BI of untreated paste without antioxidants was high at the beginning and progressively decreased during storage. This high value is in line with previous researches (Ojha et al., 2023; Tonneijck-Srpova, Venturini, Humblet-Hua, & Bruins, 2019) and it is mainly due to the low L* a* and b* detected in fresh untreated pastes, and resulting from the pre-processing applied. Mealworm larvae were indeed killed by freezing, i.e. a slow killing method responsible for enzymes activation (Leni, Caligiani, & Sforza, 2019). The long defrosting at room temperature might have further favoured the browning process. Moreover, as milling is responsible of cells breaking with release of browning enzymes, an intensification of the browning can be expected. Over time reduction of BI in untreated paste without antioxidants might instead be attributed to a progressive decolouration of melanin, i.e. the main pigment responsible for colour in edible insects (Barek, Sugumaran, Ito, & Wakamatsu, 2018). Melanin degradation is usually attributed to microorganisms such as fungi belonging to the *Aspergillus* or *Phanerochaete* genus and bacteria belonging to *Pediococcus* genus, which are able to produce peroxidases and lactases (Kim et al., 2020; Luther & Lipke, 1980; Sadaqat et al., 2020). As such microorganisms have already been isolated from insects' gut (Mamtimin et al., 2023), their presence and activity within the untreated pastes cannot be disregarded.

Addition of antioxidant to untreated pastes resulted in lower BI. Such effect has already been described in traditional food (Tao et al., 2017) and is generally associated with reduction of available oxygen for polyphenol oxidation (Golamoglu & Whitaker, 1984). However, unlike pastes U_noAOX and U_303, no BI reduction was measured during the storage of pastes U_e-Ros. This suggests that synthetic antioxidants are more effective than natural ones in scavenging the oxygen dissolved in the product, as previously observed in apple juice (Tao et al., 2017).

HHP treatment on mealworm pastes always resulted in a reduction of BI, regardless the presence of antioxidants. It may be related to inactivation of polyphenoloxidase (Wu & Yang, 2023), i.e. the enzyme primary responsible for colour degradation in insects, and to protein denaturation occurring during the treatment (Tonnejck-Srpova et al., 2019). Protein denaturation is known to be associated with syneresis and loss of water holding capacity (Ojha et al., 2023), which may lead to a reduction of the light reflectance, as observed in HHP treated fish fillets (Monteiro, Mársico, Rosenthal, & Conte, 2019).

Considering the status of the paste at day-0, samples T_noAOX and T_e-Ros showed lower BI than samples T_303, with the last having a browning level similar to samples U_303. Such observations further suggest a strong effect of the investigated food system on the antioxidant-mediated colour stabilisation. Indeed, while HHP favours the extraction of bioactive compounds naturally present in the initial tissues, it also reduces the activation energy of some chemical reaction, which might be facilitated after a pressure treatment (Medina-Meza et al., 2014). It may explain, on one hand, the lower BI measured of pastes T_e-Ros, while on the other hand, it suggests that HHP at 600 MPa for 5 min is sufficient to completely inactivate enzymes responsible for colour degradation.

Table 2. Brown index and pH of treated and untreated mealworm paste added with different antioxidants and stored at 5°C for 0 (fresh), 5, 10 and 15 days. Values (average $\pm$ standard error) within the same column followed by different lowercase letters are statistically different (p-value < 0.05). Values within the same row followed by different uppercase letters are statistically different (p-value < 0.05)

Treatment	day-0	day-5	day-10	day-15
<i>Brown index</i>				
U_noAOX	49.93 $\pm$ 3.22 a A	25.39 $\pm$ 0.55 a AB	22.84 $\pm$ 1.63 a B	22.34 $\pm$ 1.26 a B
U_303	25.72 $\pm$ 1.33 b A	25.90 $\pm$ 2.18 ab A	27.49 $\pm$ 6.43 a A	22.97 $\pm$ 4.40 a A
U_e-Ros	45.05 $\pm$ 3.58 a A	13.31 $\pm$ 2.66 c C	14.17 $\pm$ 2.34 a C	25.24 $\pm$ 0.47 a B
T_noAOX	18.30 $\pm$ 0.94 c A	20.99 $\pm$ 0.40 bc A	24.26 $\pm$ 2.49 a A	20.78 $\pm$ 0.49 a A
T_303	25.28 $\pm$ 0.79 bc A	12.87 $\pm$ 1.73 c A	15.16 $\pm$ 1.17 a A	27.81 $\pm$ 12.01 a A
T_e-Ros	18.81 $\pm$ 0.69 c A	19.00 $\pm$ 1.59 bc A	20.14 $\pm$ 4.93 a A	17.92 $\pm$ 1.38 a A

pH

U_noAOX	6.49 ± 0.03 b A	6.43 ± 0.01 c A	6.49 ± 0.01 b A	6.24 ± 0.33 a A
U_303	6.78 ± 0.00 c C	6.38 ± 0.01 b B	6.48 ± 0.01 b A	6.56 ± 0.00 a A
U_e-Ros	6.32 ± 0.07 a A	6.35 ± 0.00 a A	6.40 ± 0.01 a A	6.45 ± 0.01 a A
T_noAOX	6.87 ± 0.00 c C	6.75 ± 0.01 d B	6.64 ± 0.01 d A	6.64 ± 0.01 a A
T_303	6.87 ± 0.01 c D	6.75 ± 0.01 d A	6.57 ± 0.03 c B	6.53 ± 0.06 a C
T_e-Ros	6.85 ± 0.01 c C	6.74 ± 0.00 d BC	6.61 ± 0.01 cd AB	6.60 ± 0.02 a A

pH of mealworm paste at time 0 was close to neutrality (table 2). The addition of antioxidants seemed to have an effect only on untreated pastes, while no differences were computed on the treated ones. Irrespective of the presence of antioxidants, the treated samples always showed a higher pH than the untreated ones. It suggests buffering activity of mealworm pastes treated with HHP, which might be due to the new equilibrium between acidic and basic compounds released during the treatment (Sert & Coskun, 2022). The progressive reduction of pH observed during storage, which was common for all the samples, is in line with previous studies (Ojha et al., 2023), and might be attributed to a progressive increase of acidifying microorganisms (De Smet et al., 2019).

3.3 Effect of HHP on fatty acids profile

The fatty acid profile of the pastes is shown in table 3 and supplementary material S1. The fresh untreated pastes were characterised by high levels of unsaturated fatty acids (UFA), with oleic acid (C18:1, OA) being the prevalent, followed by palmitic (C16:0) and linoleic acids (C18:2, LA). These data are in line with previous studies (da Silva et al., 2024; Lawal, Kavle, Akanbi, Miroso, & Agyei, 2021) and confirm the high stability of mealworm fat (Rossi et al., 2022). The addition of antioxidants to the untreated pastes led to a slight increase in saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA), while monounsaturated fatty acids (MUFA) decreased (table 3). ANOSIM (PCA biplot shown in supplementary material S2) confirmed such trend, displaying a highly significant effect of the antioxidants on the fatty acid profile of untreated pastes at day-0 (ANOSIM, $p = 0.007$, $R = 0.9588$). Weaker effect was attributed to HHP (ANOSIM, $p = 0.004$, $R = 0.463$), which was however responsible for increasing SFA and MUFA (table 3). Effect of HHP on fatty acids profile has been already studied on other biomasses, although no clear trend has been observed. Indeed, while most of the investigations displayed a reduction in PUFA and an increase in MUFA and SFA (Liu, Gu, et al., 2022; Liu, Li, et al., 2022), no effects were observed on salmon (Yagiz et al., 2009) and oyster (Liu, Gu, et al., 2022), suggesting a matrix specific action. The significant increase ($p < 0.05$) in PUFA observed in the present

study, which was also observed by Ugur et al. (2021), further supports such hypothesis, leading to the conclusion that the measured differences might primarily be due to tissue modification occurring during the process.

Table 3. Fatty acid composition (% of total fatty acids) of treated and untreated mealworm paste supplemented with different antioxidants and stored at 5°C for 0 (fresh) and 15 days. Values (average $\pm$ standard error) within the same column followed by different lowercase letters are statistically different (p -value < 0.05). Values within the same row and belonging to the same parameter (SFA, MUFA, PUFA) but recorded in different days (day-0, day-15) followed by different uppercase letters are statistically different (p -value < 0.05).

Treatment	day-0			day-15		
	SFA	MUFA	PUFA	SFA	MUFA	PUFA
U_noAOX	34.94 $\pm$ 0.29 bc B	48.50 $\pm$ 0.38 a A	16.56 $\pm$ 0.19 a B	43.82 $\pm$ 0.16 a A	36.02 $\pm$ 0.19 c B	20.16 $\pm$ 0.03 a A
U_303	36.97 $\pm$ 1.06 bc B	45.07 $\pm$ 0.38 ab A	17.96 $\pm$ 0.80 b	41.97 $\pm$ 0.80 a A	37.21 $\pm$ 0.16 bc B	20.82 $\pm$ 0.65 a
U_e-Ros	40.13 $\pm$ 2.37 ab	39.47 $\pm$ 1.54 c A	20.41 $\pm$ 0.91 c	43.51 $\pm$ 0.57 a	34.56 $\pm$ 0.74 c B	21.93 $\pm$ 1.08 a
T_noAOX	34.16 $\pm$ 0.43 c B	44.02 $\pm$ 0.33 b A	21.82 $\pm$ 0.11 e	42.25 $\pm$ 2.01 a A	38.35 $\pm$ 1.91 bc B	19.40 $\pm$ 2.13 a
T_303	43.11 $\pm$ 1.07 a A	36.03 $\pm$ 1.09 c B	20.86 $\pm$ 0.13 d A	38.84 $\pm$ 0.22 ab B	41.43 $\pm$ 0.26 ab A	19.73 $\pm$ 0.09 a B
T_e-Ros	40.56 $\pm$ 0.96 ab A	39.50 $\pm$ 1.15 c B	19.93 $\pm$ 0.20 bc	34.05 $\pm$ 1.34 b B	45.40 $\pm$ 0.98 a A	20.56 $\pm$ 0.36 a

Important differences were observed between samples U_noAOX and samples U_e-Ros (table 3), i.e. the pastes containing the antioxidant derived from rosemary. The same trend was not observed in treated pastes, where lower PUFA and MUFA and higher SFA were detected in sample T_noAOX than in samples with antioxidants (table 3).

Considering the 15 days of refrigerated storage, an increase in SFA (especially stearic acid) and a decrease in MUFA (especially oleic acid) was noticed in all the untreated pastes, regardless by the presence of antioxidants (table 3 and supplementary material S1). Slight increase in PUFA (especially linoleic acid) was also observed. Similar results have been reported in other foods, including frozen pork (Alonso et al., 2016), dried beef (Torres, Pearson, Gray, & Ku, 1989), goose meat (Orkusz, Wolanska, & Krajinska, 2021), and raw fish (de Castro et al., 2007), and it is generally associated to enzymatic hydrolysis of phospholipids occurring during the storage as well as to a progressive oxidation of the fat. A similar evolution of the fatty acid profile was also observed in samples T_noAOX, while treated pastes with antioxidants exhibited a completely different trend, with SFA decreasing and MUFA increasing during storage (table 3). This suggests that the antioxidants might have prevented lipolytic and oxidative reactions happening during the storage, although their activity was only explicated after HHP treatment. Further, HHP may have favoured a better contact between fatty acids and antioxidant compounds (Medina-Meza et al., 2014), resulting in an enhanced protective effect.

3.4 Effect of HHP on fat oxidation

Effect of HHP on fat oxidation is shown in table 4. AV measured on the fat extracted from the pastes showed that HHP was responsible for a general reduction of free fatty acids. Such reduction, which was independent from the presence of antioxidants, might be explained by an inactivation of the lipolytic enzymes present within the pastes (Gao, Yang, Rong, Bao, & Zhang, 2014). However, increase of AV was observed in all the samples during the storage, with the treated pastes always showing lower AV than untreated pastes. It suggests that HHP at 600 MPa for 5 min was not able to permanently inactivate the lipases present in *T. molitor* larvae on one hand, while on other hand led to conclude that HHP might be a strategy for elongating the shelf life of mealworm pastes. No apparent effect of antioxidants was detected.

Table 4. Acid value (mg KOH/g fat), peroxide value (meq O₂/kg fat) and TBARs (μg MDA/kg sample) of treated and untreated mealworm paste supplemented with different antioxidants and stored at 5°C for 0 (fresh), 5, 10 and 15 days. Values (average ± standard error) within the same column followed by different lowercase letters are statistically different (p-value < 0.05). Values within the same row followed by different uppercase letters are statistically different (p-value < 0.05)

Treatment	day-0	day-5	day-10	day-15
<i>Acid value (mg KOH/g fat)</i>				
U_noAOX	74.81 ± 9.72 ab B	99.74 ± 3.47 a B	95.38 ± 3.24 a B	126.33 ± 2.75 a A
U_303	72.94 ± 1.08 b B	99.74 ± 1.65 a B	101.61 ± 0.62 a A	116.57 ± 3.30 a A
U_e-ROS	76.05 ± 1.25 a C	104.11 ± 1.25 a B	99.12 ± 2.16 a B	114.70 ± 1.25 ab A
T_noAOX	53.61 ± 1.65 c C	54.86 ± 2.72 b BC	76.68 ± 1.08 b B	91.64 ± 8.64 c A
T_303	53.61 ± 1.65 c C	57.98 ± 2.16 b B	63.59 ± 0.00 c B	95.38 ± 1.87 bc A
T_e-ROS	56.11 ± 4.71 c C	57.98 ± 1.87 b BC	69.82 ± 0.62 bc B	84.78 ± 3.12 c A
<i>Peroxide value (meq O₂/kg fat)</i>				
U_noAOX	11.11 ± 2.94 ab A	9.58 ± 4.68 a A	17.78 ± 1.11 b A	15.56 ± 4.01 b A
U_303	13.78 ± 1.11 a AB	4.44 ± 1.11 c C	17.78 ± 1.11 b AB	22.22 ± 4.84 a A
U_e-Ros	13.78 ± 2.94 a B	8.89 ± 1.11 ab C	20.00 ± 0.00 a A	3.33 ± 0.00 d D
T_noAOX	4.53 ± 0.38 b A	11.11 ± 1.11 a A	4.44 ± 1.11 d A	8.89 ± 1.11 c A
T_303	2.42 ± 0.11 b B	3.33 ± 0.00 c B	14.44 ± 1.11 c A	23.33 ± 1.92 a A
T_e-Ros	12.22 ± 2.22 a A	5.56 ± 1.11 bc BC	3.33 ± 0.00 d C	8.89 ± 5.56 c AB
<i>TBARs (μg MDA/kg sample)</i>				
U_noAOX	277.45 ± 3.94 ab A	289.97 ± 3.39 a A	247.55 ± 2.83 b B	274.24 ± 4.03 b A
U_303	240.81 ± 4.50 d B	315.03 ± 8.57 a A	244.45 ± 6.76 b A	239.14 ± 5.27 c B
U_e-Ros	288.01 ± 8.11 a AB	316.35 ± 11.34 a A	288.19 ± 8.99 a AB	262.75 ± 5.04 bc B
T_noAOX	254.65 ± 2.00 bcd B	297.38 ± 7.21 a A	298.55 ± 4.09 a A	274.29 ± 2.29 b A
T_303	249.55 ± 2.76 cd B	286.75 ± 3.33 a A	278.79 ± 3.23 a B	303.16 ± 7.79 a A

T _e -Ros	272.12 ± 5.54 abc C	308.30 ± 6.01 a A	293.80 ± 2.63 a B	304.81 ± 8.18 a A
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HHP was found to promote POV reduction in all the pastes except T_e-Ros. Such observation, might have two suitable explanations, i.e. the promotion of secondary oxidation reactions (HHP is known for retarding the formation of radicals and enhancing the reaction between radicals (Tauscher, 1995)) and the enhanced extraction of the natural antioxidants contained in the larvae (Navarro-Baez, Martínez, Welte-Chanes, Buitimea-Cantúa, & Escobedo-Avellaneda, 2022). It is therefore hypothesized that, similarly to fish and contrary to meat, higher pressures are responsible for reduction in oxidation. Considering the evolution of POV along the storage, high stability was observed for antioxidant free pastes. On the other hand, pastes with synthetic antioxidant displayed low POV at the beginning and high POV after 10 days of storage. It suggests that, although the synthetic antioxidant explicates high and fast activity, its effect was limited in time. On the other hand, no effect of the natural antioxidant was noticeable at time 0, while significant lower POV were measured for these pastes at day-5, 10 and 15. Therefore, activity of the natural antioxidant was slower but longer than the activity of the synthetic antioxidant. Similar results have also been observed in HHP treated chicken and beef meat, where synthetic antioxidants showed high ability in reducing the radical formation during pressurisation, while rosemary extracts were more active during the storage (Bolumar, Andersen, & Orlie, 2011, 2014). HHP treatment also led to TBARs reduction. This date contradicted the results obtained in a previous study (Ojha et al., 2023), where a rapid increase in TBARs was observed after HHP treatment. Possible explanation for this discrepancy can be the status of the initial material, as well as the pre-process applied. Indeed, although HHP is often associated to lipid oxidation (Medina-Meza et al., 2014), it does not directly induce it, as several factors, including intrinsic properties of the food, presence of pro-oxidant and antioxidant molecules, presence of enzymes and level of oxygen, appear crucial. For example, lower oxidation has been observed in fish submitted to HHP if vacuum packaging was applied (Monteiro, Rosário, Neto, & Conte-Junior, 2024). As in the present study, mealworm pastes were packed under vacuum, absence of oxygen can be assumed, during the overall experiment. On the other hand, in the study carried out by Ojha et al. (2023), the packages were open immediately after the treatment. Therefore, early contact with oxygen could not be denied, resulting in high initial oxidation.

Considering the storage period, stable TBARs were noticed in both, U_noAOX and T_noAOX pastes. Slight increase was instead observed in pastes containing antioxidants. Such increase was marked at day-5 for untreated pastes, while it was lower, although still present, in treated pastes. This difference can be explained by a partial inactivation of the lipoxygenase enzymes during the HHP treatment (Monteiro et al., 2024), which was however reversed during the storage. The progressive degradation in TBARs observed during the following days, further suggests that untreated vacuum packaged pastes were stable, while HHP treated paste supplemented with antioxidants could still have some stability problems, possibly due to an early consumption of antioxidant (supplementary material S3) or to a negative combination of the used antioxidants with HHP (H. J. Ma, Ledward, Zamri, Frazier, & Zhou, 2007).

3.5 Effect of HHP on fluorescence spectra

Fluorescence spectroscopy has been described as a rapid method for detecting protein and lipid oxidation in food (Barriuso, Astiasarán, & Ansorena, 2013; Domínguez et al., 2022). Several excitation/emission couples has been identified for carrying interesting information, including 280/330-350 nm which is typically associated to tryptophan and 350/400-500 nm, corresponding to Schiff bases (Domínguez et al., 2022). However, due to the complex chemical structure of the material and to the interaction of several molecules, different wavelengths might need to be considered in different food.

Excitation wavelengths ranged between 280 and 290 nm, 320 and 350 nm, 380 and 390 nm were studied in the present experiment. PARAFAC analyses carried out on the overall spectra as well as on the region-specific spectra showed the presence of three main peaks, centred at excitation/emission of 280/330-332 nm, 285/340-380 nm and 330/385-440 nm (supplementary material S4). According to Ojha et al. (2023), peaks recorded at excitation 280 and 285 nm are both associated to tryptophan. On the other hand, fluorescence peak recorded at 330 nm might be associated to Schiff bases produced from the reaction between aldehydes (resulting from the lipid oxidation) and amino groups of protein (Christensen, Povlsen, & Sorensen, 2003; Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008). Tryptophan fluorescence displayed a progressive shift toward higher emission wavelengths, suggesting a progressive protein unfolding with tryptophan being exposed to hydrophilic environment. Such shift has been already noted in previous experiments (Boukil, Marciniak, Mezdour, Pouliot, & Doyen, 2022;

Bußler, Rumpold, Jander, Rawel, & Schlüter, 2016; Estévez et al., 2008; Ojha et al., 2023). However, while differences in peak location were also observed after 5 days of refrigerated storage, they disappeared after a longer period of time, suggesting that the effect of HHP on protein structure was only limited to few days after the treatment.

No differences were observed after HHP on tryptophan peak intensity (table 5). Higher differences were instead observed during the storage, with treated samples always showing lower fluorescence than untreated ones.

Increase in tryptophan fluorescence intensity was observed in all the untreated samples during storage. On the other hand, stable fluorescence intensity was measured for the treated pastes. These differences in behaviour might be due to an irreversible degradation of the protein happening during the HHP treatment, and already described by Boukil et al. (2022). The fluorescence intensity recorded at excitation 330 nm further confirmed such observation. Higher intensity was indeed measured for samples T at time 0 and 5. In this case, however, the trend was quite linear, with samples T_noAOX displaying the highest peak, followed by T_303, T_e-Ros, U_noAOX, U_303 and U_e-Ros. This further suggests that HHP contributed to the oxidative degradation of the product; however, this effect might be controlled by the addition of antioxidants. A reverse trend was instead observed on day-15, suggesting a progressive degradation of this fluorophore, as well as a deterioration in the oxidative stability of untreated pastes (Hassoun & Karoui, 2016; Veberg et al., 2006).

Table 5. Fluorescence PARAFAC component scores calculated at different wavelengths (component 1: excitation wavelength = 280nm; component 2: excitation wavelength = 285nm; component 3: excitation wavelength = 330nm) of treated and untreated mealworm paste supplemented with different antioxidants and stored at 5°C for 0 (fresh), 5, 10 and 15 days. Values (average $\pm$ standard error) within the same column followed by different lowercase letters are statistically different (p-value < 0.05). Values within the same row followed by different uppercase letters are statistically different (p-value < 0.05)

Treatment	day-0	day-5	day-10	day-15
<i>Fluorescence-PARAFAC score 280nm</i>				
U_noAOX	1115.43 $\pm$ 43.38 a B	1279.52 $\pm$ 13.19 a AB	1346.35 $\pm$ 27.57 a A	1406.26 $\pm$ 55.68 a A
U_303	1200.08 $\pm$ 47.15 a B	1268.61 $\pm$ 41.16 a AB	1348.29 $\pm$ 19.84 a AB	1369.76 $\pm$ 16.03 a A
U_e-Ros	1054.49 $\pm$ 23.91 a C	1189.05 $\pm$ 14.89 a B	1307.62 $\pm$ 16.09 a A	1405.57 $\pm$ 39.67 a A
T_noAOX	1029.18 $\pm$ 67.33 a A	1055.28 $\pm$ 7.38 b A	1032.07 $\pm$ 4.57 b A	1025.33 $\pm$ 38.33 b A
T_303	1036.30 $\pm$ 51.54 a A	993.98 $\pm$ 23.25 bc A	1009.39 $\pm$ 27.77 bc A	929.72 $\pm$ 7.63 b A
T_e-Ros	985.47 $\pm$ 26.85 a A	942.44 $\pm$ 4.51 c AB	917.44 $\pm$ 31.10 c AB	868.21 $\pm$ 14.87 b B

Fluorescence-PARAFAC score 285nm

U_noAOX	496.71 ± 11.54 a C	588.33 ± 9.65 a B	617.82 ± 13.07 a AB	660.98 ± 19.77 a A
U_303	510.45 ± 7.05 a C	562.06 ± 12.28 ab B	595.95 ± 6.99 a AB	601.06 ± 0.27 ab A
U_e-Ros	478.15 ± 7.00 a D	529.40 ± 14.02 b C	572.98 ± 3.93 ab B	649.07 ± 8.85 a A
T_noAOX	573.87 ± 45.92 a A	590.49 ± 4.33 a A	574.51 ± 8.31 ab A	544.72 ± 26.86 bc A
T_303	547.90 ± 31.40 a A	538.33 ± 10.07 b A	566.14 ± 14.60 ab A	494.84 ± 7.93 cd A
T_e-Ros	540.74 ± 13.17 a A	535.28 ± 4.39 b A	536.75 ± 20.12 b A	463.94 ± 9.57 d B

Fluorescence-PARAFAC scores 330nm

U_noAOX	376.39 ± 5.76 bc B	445.03 ± 12.45 bc A	450.84 ± 11.18 a A	489.98 ± 22.07 a A
U_303	401.21 ± 0.27 bc A	440.61 ± 7.08 bc A	427.96 ± 16.21 a A	424.28 ± 8.09 bc A
U_e-Ros	349.61 ± 4.32 c C	414.42 ± 5.28 c B	427.29 ± 4.69 a B	483.93 ± 9.68 ab A
T_noAOX	498.52 ± 41.92 a A	491.05 ± 2.33 a A	460.70 ± 3.38 a B	404.33 ± 16.96 cd C
T_303	457.02 ± 25.22 ab A	453.04 ± 6.95 ab A	479.52 ± 14.03 a A	369.47 ± 8.26 cd B
T_e-Ros	468.11 ± 6.52 ab A	461.20 ± 10.13 ab A	451.22 ± 14.58 a A	350.65 ± 9.45 d B

3.6 Effect of HHP on microbial status

Microbial contamination of mealworm pastes is shown in figure 2. Significant reduction of counts (2.7-3.4 log CFU/g for TVC-aerobic, 4.5 log CFU/g for TVC-anaerobic, 5 log CFU/g for LAB) were always detected after HHP. This is in line with previous experiments conducted on edible insect products as well as on more traditional food (Evrendilek, 2022; Ojha et al., 2023; Rumpold et al., 2014). However, it should be mentioned that higher microbial reduction is usually found on meat, juices and other foods when HHP was applied (Rendueles et al., 2011). The main reason for such low inactivation might be found on the high water activity as well as on the high initial microbial counts of the product (Borremans, Smets, & Van Campenhout, 2020; Wynants et al., 2019). Addition of antioxidants resulted in lower initial counts, indicating antimicrobial activity of the antioxidants. For instance, rosemary extracts are known for being active against Gram negative bacteria (Nieto, Ros, & Castillo, 2018), such as *Enterobacteriaceae* (i.e. the most abundant microbial family in the insect gut (Osimani et al., 2018)). On the other hand, antimicrobial activity observed on the 303 samples might be mainly due to citric acid as well as to a synergistic effect of propyl gallate and BHA (Burel, Kala, & Purevdorj-Gage, 2021; Eliuz, 2020; Shih & Harris, 1977).

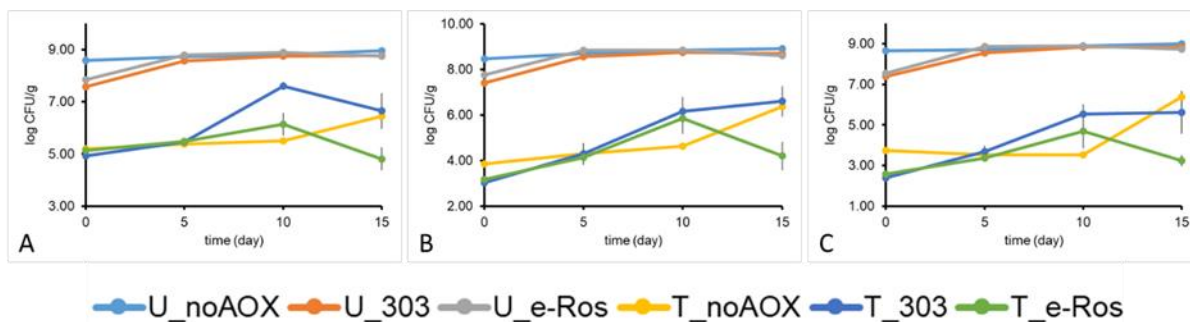


Figure 2. Microbial contamination (log CFU/g: A. total viable aerobic count; B. total viable anaerobic mesophilic count; C. lactic acid bacteria) of treated and untreated mealworm paste mixed with several antioxidants, stored at 5°C for 0 (fresh), 5, 10 and 15 days.

Contamination levels of U_noAOX pastes appeared to be stable during the storage, and always higher than other pastes. Initial differences between antioxidant supplemented and not-supplemented pastes tended to flatten out, disappearing after 5 days of storage. It may be due to a progressive consumption of the antioxidants, which led to a reduction of their antimicrobial activity. Pairwise comparison of antioxidant recovery (supplementary material S3) supported this speculation with recovery of tocopherol and propyl-gallate measured at day-15 being significantly lower than at day-0.

General increase in microbial counts was also observed for samples T. However, while slow increase was measured for samples T_noAOX during the first 10 days, fast increase was detected afterwards. It suggests that 10 days is the ideal shelf-life of HHP treated mealworm pastes.

No differences between treated pastes with and without antioxidant were computed after 5 days of refrigerated storage. Significant differences were instead measured at day-10, with antioxidant supplemented pastes having higher contamination. It further supports the idea that antimicrobial effect of antioxidants weakened as the storage time increased.

4. Conclusions

The present research shows that fresh mealworm paste can be treated by HHP, ensuring acceptable microbial reduction and chemical stability with a suggested shelf life of at least 10 days under refrigerated conditions. HHP did not display any noticeable effect on lipid oxidation, although the

application of vacuum during the overall experiment may have hampered the results. Similarly, since the investigated storage period was limited to 15 days, no complete absence of oxidation could be assumed over longer time. No critical effect of the antioxidants was observed in this experiment, although differences between the pastes with or without antioxidants were evident. Synthetic antioxidant explicitly showed high antioxidant activity during and immediately after HHP treatment. On the other hand, natural antioxidants appeared to be more active over a longer period of time. Consequently, appropriate mixtures of the two antioxidants might be a solution to maximize their effects. Future research should investigate the effect of different antioxidant blends, as well as of different packaging and longer storage times on the physio-chemical stability of mealworm pastes. Study of alternative processing technologies as well as different HHP parameters also requires more attention.

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A8. Fresh aquaculture sludge management with black soldier fly (*Hermetia illucens* L.) larvae: investigation on bioconversion performances

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OPEN Fresh aquaculture sludge management with black soldier fly (*Hermetia illucens* L.) larvae: investigation on bioconversion performances

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Aquaculture solid waste (ASW) is a nutrient rich material that can pose a significant environment challenge if not properly managed. This study investigated the potential of black soldier fly (BSF) larvae in converting this waste into biomass. Five substrates comprising chicken feed supplemented with varying proportions of fresh ASW (0%, 25%, 50%, 75%, 100%) were formulated and evaluated for larval growth and waste bioconversion efficiency. High nutrients retention (N: $23.25 \pm 1.40\%$; C: $21.94 \pm 0.99\%$; S: $12.20 \pm 1.33\%$) and feed conversion ratio (1.78 ± 0.08) were detected on substrate 100ASW, although the limited feeding rate (114.54 ± 5.38 mg dry substrate/larvae) and the high amount of indigestible fibres (ADF = $15.87 \pm 0.24\%$; ADL = $6.36 \pm 0.17\%$) were translated to low larval growth (final larval average weight: 66.17 ± 1.81 mg). Decreasing ASW content resulted in reduced fibres and ash, increase in non-fibrous carbohydrates and C/N ratio, and improved larval growth and substrate utilization. However, high larval metabolic activity suggested higher nutrients loss to the environment. Substrate 75ASW demonstrated the best performances in terms of larval production (final larval average weight: 176.30 ± 12.12 mg), waste reduction (substrate reduction corrected by percentage of ASW: $26.76 \pm 0.86\%$) and nutrients assimilation (N: $22.14 \pm 1.14\%$; C: $15.29 \pm 0.82\%$; S: $15.40 \pm 0.99\%$). This substrate closely aligned with optimal BSF rearing substrates reported in literature. Overall, this study highlights the potential of BSF larvae in managing fresh ASW, offering a dual benefit of waste reduction and insect biomass production.

Aquaculture solid waste (ASW) management is one of the biggest environmental problems connected with the aquaculture sector¹. Such waste (a.k.a. aquaculture sludge) is mainly composed of fish carcasses, uneaten feed, faecal drops and metabolic products², and is rich in nitrogen, phosphorous and organic matter³. It can potentially be a serious threat for both, fish rearing performances and environmental sustainability. If not readily removed, such material may accumulate in the rearing pond causing eutrophication and generating greenhouse gas emission^{4,5}.

Traditional intensive and semi-intensive aquaculture systems have low efficiency in terms of nutrient assimilation and recovery, resulting in high concentration of organic matter, nitrogen and phosphorous in the rearing water¹. Innovative technologies, such as integrated multi-trophic aquaculture, aquaponic, biofloc technology and recirculating aquaculture system (RAS), which have the aim of solving these problems have been implemented¹. The principle behind such systems is to use the aquaculture rearing waste and/or recycle the effluent water, which is cleaned, disinfected, re-oxygenated and reintroduced in the rearing pond. Although these technologies can significantly reduce the amount of effluent water discarded from the system, they cannot solve the production of aquaculture solid waste².

One solution for combining waste treatment along with nutrient recovery may be the bioconversion of aquaculture sludge by insects. Black soldier fly (BSF, *Hermetia illucens*) larvae have been reported for being able to efficiently grow on organic wastes, such as manure and food waste, converting them in high quality proteins to

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be used as animal feed^{6,7}. Additionally, the residual material after insect rearing (frass) may still be conveniently used as organic fertiliser in agriculture⁸, thus promoting a transition to the circular economy.

Despite the growing interest in using insects for waste recycling, only few studies have investigated the ability of BSF larvae in bio-converting ASW, so far. In a study from Schmitt et al.⁹, oven-dried aquaculture sludge from salmon farming was regenerated at 70% of moisture and tested for BSF larvae rearing. Although the authors concluded that ASW was suitable for BSF growth, insects' performances were particularly low, suggesting the need to add other ingredients to the rearing substrate. A recent study from Liland et al.¹⁰ has shown that addition of chicken feed to the oven dried salmon sludge could ensure acceptable larval growth with a final larval weight stable at levels of sludge inclusion as high as 40%. However, the drying process applied to the sludge may have had a strong impact on the substrate quality, hiding the real potential of ASW for rearing BSF larvae. Moreover, the oven drying is an energy costly pre-process, which may limit the technical applicability of the material.

In the present study, the ability of BSF larvae to reduce and convert fresh ASW from pikeperch (*Sander lucioperca*) production was investigated. Intermediate aquaculture sludge discarded from the drum filter (i.e. before the bacterial treatment for NH₃ removal) of a pilot scale RAS was mixed with commercial chicken feed (a common rearing substrate for BSF larvae¹¹) in different ratios and larval growth and bioconversion performances were evaluated. This research aims to evaluate BSF larval growth and bioconversion under varying ASW/chicken feed mixtures ratios, contributing valuable insights to the sustainable ASW management.

Results

Chemical composition of substrates

All the tested substrate, except 100ASW, showed a similar DM with values ranged between 65 and 70%. Substrate 100ASW contained significantly lower DM, with a moisture level of $92.05 \pm 0.10\%$ (Table 1).

In terms of composition (Fig. 1), CF acted as the main source of carbohydrates, with the 0ASW diet exhibiting an NFC level of $58.17 \pm 0.91\%$, which was similar to substrates 25ASW and 50ASW, but significantly higher than treatment 75ASW and 100ASW ($P = 0.006$). Inclusion of ASW in diet led to a progressive increase in ash (Beta regression, $P = 0.006$), hemicellulose ($P < 0.001$), cellulose ($P < 0.001$), and lignin ($P = 0.006$). Lipid contents were found similar in 25ASW, 50ASW, 75ASW, 100ASW and slightly lower value was observed in 0ASW

Treatment	ASW (%)	CF (%)	ASW (g)	CF (g)	Water (g)	Total wet weight (g)	Dry matter (% fresh matter)*
0ASW	0	100	0	99.47	200.53	300.00	36.53 ± 0.13
25ASW	25	75	41.76	125.28	132.96	300.00	30.23 ± 0.12
50ASW	50	50	117.31	117.31	65.38	300.00	29.10 ± 0.04
75ASW	75	25	225	75.00	0.00	300.00	29.94 ± 0.66
100ASW	100	0	300	0.00	0.00	300.00	7.95 ± 0.06

Table 1. Composition and final dry matter (% of fresh matter) of the experimental substrates. *Dry matter is expressed as mean $\pm$ standard error of 3 repetitions. ASW aquaculture solid waste, CF chicken feed.

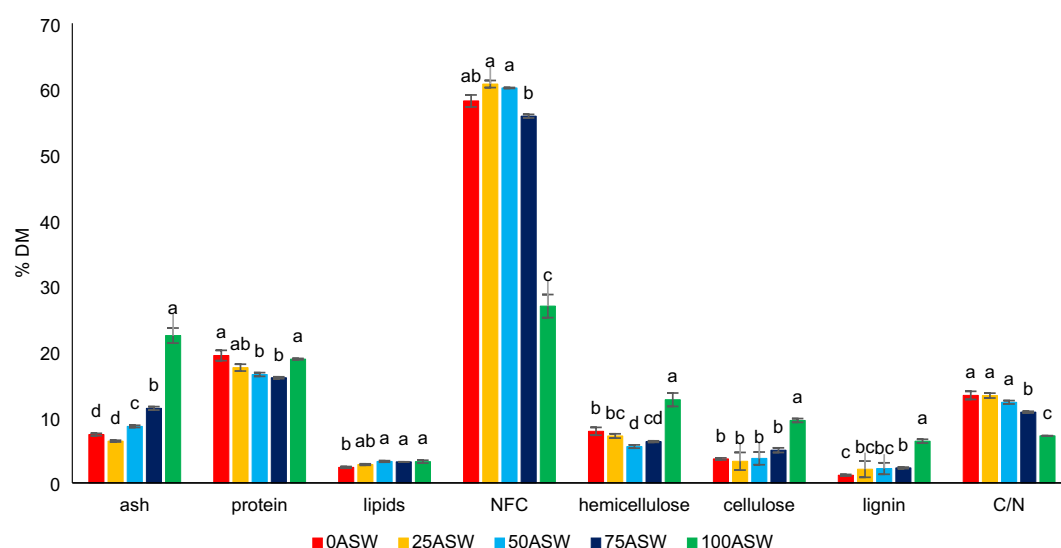


Figure 1. Chemical composition (% DM) of diets used for BSF larvae rearing. Error bars correspond to the standard error computed on 3 replicates/treatment. Different letters on top of the bars within the same parameters indicate significant difference ($P < 0.05$).

($P=0.003$). Carbon/Nitrogen ratio gradually decreased from approximately 13 (0ASW, 25ASW, 50ASW) to 10.78 (75ASW), reaching to 7.17 in the substrate 100ASW ($P=0.006$). The estimated level in proteins was similar in 0ASW ($19.40 \pm 0.79\%DM$) and 100ASW ($18.85 \pm 0.10\%DM$), although some differences were detected between these two substrates and treatments 25ASW ($17.56 \pm 0.52\%DM$), 50ASW ($16.51 \pm 0.28\%DM$) and 75ASW ($16.01 \pm 0.14\%DM$) ($P=0.001$).

Larval growth and bioconversion performances

Larval performances in terms of ability to grow, convert the diet in larval body mass and reduce the overall substrate, are presented in Table 2. Inclusion of ASW in diet did not showed any adverse effect on larval survival, with value ranged between 75.83 (25ASW) and 89.00% (50ASW). Slower development was observed for larvae housed on substrates 75ASW and 100ASW ($P=0.002$), where the first pre-pupae was observed 2 days later than in the other substrates.

Average gained weight (GW) ranged between 0.06 and 0.08 g, except for treatment 75ASW where an increase of 0.17 g was observed ($P<0.001$). Trend of weight increase overtime was similar in all the treatments (Fig. 2), with an exponential tendency observed during the first 11 (0ASW, 25ASW, 50ASW) or 13 days (75ASW), followed by a stationary (50ASW) or decreasing phase. Two steps growth was observed in treatment 100ASW, where larvae raised faster during the first 9 days and slower between day 9 and day 11.

As shown in Table 2, treatment 75ASW resulted in significantly higher BCR than the other treatments ($P<0.001$), with double value than the control (0ASW). A clear decreasing trend was observed for AF and FCR with an increasing percentage of ASW inclusion in the diet, which decreased from 47.64 g and 14.08 (0ASW) to 5.28 g and 1.78 (100ASW), respectively. No clear trend was observed in terms of substrate reduction, with treatments 0ASW (43.43%) and 75ASW (35.68%) showing significantly higher values than the other substrates ($P<0.001$).

Larval activity on the substrate led to reduction in DM, VS, hemicellulose, cellulose, C, TOC, N, S and P (Table 3). The highest reductions were always observed in treatments 0ASW and 75ASW, regardless the considered nutrient. High reduction in K and NO_3-N were also observed, while concentration of NH_4-N increased in all the treatments, except in treatment 100ASW where a reduction of 75% (fresh matter bases) was noticed. pH increasing was observed in all the treatments. Initial substrates had a pH ranged between 4.89 and 6.63, with substrate 100ASW showing the highest pH. The lowest pH was detected in substrate 25ASW, followed by substrate 50ASW (4.92), 75ASW (5.23) and 0ASW (5.99). Regardless of the treatment, pH of the final material

Parameter	0ASW	25ASW	50ASW	75ASW	100ASW
Survival (%)	83.00 $\pm$ 4.80	75.83 $\pm$ 1.83	89.00 $\pm$ 1.53	84.50 $\pm$ 5.00	88.74 $\pm$ 1.13
Development (days)	8.00 $\pm$ 0.00	9.33 $\pm$ 0.67	8.67 $\pm$ 0.67	10.00 $\pm$ 0.00*	10.00 $\pm$ 0.00*
GW (mg)	0.07 $\pm$ 0.01 b	0.06 $\pm$ 0.00 b	0.08 $\pm$ 0.01 b	0.17 $\pm$ 0.01 a	0.06 $\pm$ 0.00 b
AF (g)	47.64 $\pm$ 2.42 a	25.49 $\pm$ 2.68 b	21.22 $\pm$ 2.44 b	32.14 $\pm$ 1.75 b	5.28 $\pm$ 0.07 c
FCR	14.08 $\pm$ 2.69 a	9.18 $\pm$ 1.84 ab	4.55 $\pm$ 0.89 bc	2.80 $\pm$ 0.08 bc	1.78 $\pm$ 0.08 c
BCR (%)	4.04 $\pm$ 0.72 b	3.23 $\pm$ 0.30 b	4.89 $\pm$ 0.44 b	9.68 $\pm$ 0.52 a	3.92 $\pm$ 0.20 b
Reduction (%)	43.43 $\pm$ 2.10 a	28.07 $\pm$ 2.92 bc	23.88 $\pm$ 2.79 bc	35.68 $\pm$ 1.14 ab	22.11 $\pm$ 0.16 c

Table 2. Growth and bioconversion indexes of BSF larvae reared on the experimental diets (mean $\pm$ standard error; $n=3$). ASW aquaculture solid waste, GW gained weight, AF assimilated feed, FCR feed conversion ratio, BCR biomass conversion rate. Values within a row followed by different letters are significant different ($P<0.05$). Survival and development values followed by a star (*) are significant different from the control (0ASW) (GLM + Dunnett's test). All the measured parameters are referred to the final larvae.

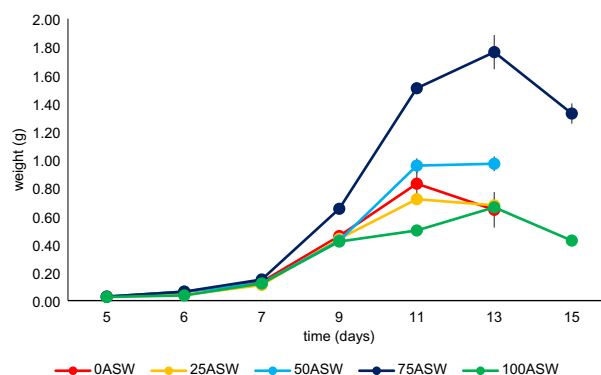


Figure 2. Averaged weight increase trend of 10 BSF larvae reared on the experimental substrates. Error bars correspond to the standard error computed on 3 replicates/treatment.

Parameter	0ASW	25ASW	50ASW	75ASW	100ASW
VS	43.16 ± 2.26 a	30.46 ± 2.17 b	27.68 ± 2.65 b	38.89 ± 0.87 ab	30.65 ± 1.34 ab
N	45.16 ± 5.43 ab	19.82 ± 2.67 b	33.38 ± 9.16 ab	54.68 ± 0.50 a	40.88 ± 0.44 ab
C	43.74 ± 1.93 a	31.41 ± 2.55 b	29.12 ± 2.53 b	40.91 ± 0.75 ab	34.34 ± 0.39 ab
S	40.37 ± 1.61 a	13.64 ± 2.13 c	20.18 ± 6.96 bc	30.19 ± 0.56 ab	22.67 ± 0.97 abc
TOC	42.10 ± 2.36 a	28.14 ± 4.09 ab	26.26 ± 3.59 b	42.74 ± 2.06 a	34.72 ± 1.05 ab
NH ₄ -N	-204.66 ± 31.32 c	-96.70 ± 28.63 b	-93.15 ± 4.46 b	-33.46 ± 1.38 b	75.28 ± 0.90 a
NO ₃ -N	82.29 ± 3.55 ab	60.92 ± 2.09 b	69.47 ± 3.60 b	87.83 ± 2.45 a	95.82 ± 0.57 a
P	28.58 ± 6.43 a	6.50 ± 1.69 b	4.82 ± 4.91 b	6.48 ± 2.45 b	5.57 ± 1.40 b
K	84.52 ± 2.14 b	89.17 ± 0.42 b	87.22 ± 0.14 b	89.38 ± 1.04 b	99.70 ± 0.01 a
Hemicellulose	37.16 ± 4.22 a	22.02 ± 2.46 ab	15.56 ± 3.65 b	23.43 ± 1.16 ab	3.71 ± 0.57 c
Cellulose	27.45 ± 7.43 a	15.65 ± 2.29 b	10.36 ± 0.12 b	17.75 ± 1.86 b	9.93 ± 1.02 b
Lignin	27.93 ± 2.54 a	-18.79 ± 8.85 c	11.35 ± 4.55 b	22.01 ± 3.89 a	-0.79 ± 0.38 c
pH*	1.04 ± 0.19 b	1.73 ± 0.13 a	2.30 ± 0.22 a	2.00 ± 0.06 a	0.70 ± 0.02 b

Table 3. Reduction in chemical fractions (% of fresh matter) and pH changes after the BSF-mediated substrate bioconversion. ASW aquaculture solid waste, VS volatile solids, N total nitrogen, C total carbon, S total sulphur, TOC total organic carbon, NH₄-N ammonium nitrogen, NO₃-N nitric nitrogen, P elemental phosphorous, K elemental potassium. Values within a row followed by different letters are significant different (P < 0.05). *pH changes computed as difference between pH of frass and pH of substrates.

was close to neutral, being ranged between 6.62 (25ASW) and 7.33 (100ASW). pH 7 (7.03) was observed in frass 0ASW, while similar pH was recorded from frass 50ASW (7.22) and 75ASW (7.33).

The mass balance of nitrogen, carbon and sulphur and relative nutrient retention from insects are shown in Fig. 3. Irrespective by the nutrient, the highest retention was always detected in treatment 75ASW followed by treatment 100ASW, whose values were significantly higher than the other treatments (ANOVA, P < 0.05). No statistically significant differences were observed between 0ASW, 25ASW and 50ASW. The amounts of lost nutrients were conspicuously higher in treatment 0ASW than in the others, except for nitrogen where no differences were detected between 0ASW (35.44%) and 75ASW (32.53%). Lower nitrogen loses were observed in treatments 50ASW (20.37%) and 100ASW (23.25%), while substrate 25ASW presented very limited loses (9.25%).

Nutritional composition of insects

Chemical composition of BSF larvae growth on the tested substrates is shown in Table 4. DM was always close to 30%, with the only exception of larvae 75ASW and 100ASW, which showed significantly higher and lower DM (P < 0.001). VS appeared for being stable among the different treatments containing CF, with significantly lower values only detected in treatment 100ASW (P < 0.001). Protein, lipids and NFC varied according with the treatment, with the highest values detected in larvae 25ASW for protein, 50ASW and 75ASW for lipids, 75ASW and 100ASW for NFC, while significantly lower values were measured in larvae 75ASW for protein (P = 0.006) and 100ASW for lipids (P < 0.001). Fibres content was stable between treatments, except for larvae 100ASW, which presented a significantly lower amount of NDF (P = 0.012) and higher amount of ADL (P = 0.038). No statistically relevant differences were observed in ADF concentration.

Nutritional composition of frass

Table 5 displays the chemical composition of frass. Although the substrates had different DM, no significant differences were detected on DM between frass from the different treatments (P = 0.122). Compared with the others, frass from treatment 100ASW appeared for being significant poorer in VS (72.70%), C (38.60%), TOC (34.23%) and K (0.90 g/kg FM). On the other hand, the same frass were richer in fibres, N (4.85%) and P (25.62 g/kg FM). No significant differences were detected on NO₃-N, while significant higher amount of NH₄-N was recorded in treatment 75ASW (5003.94 mg/kg FM) than in the other treatments (P < 0.001). The lowest amount of NH₄-N was measured in frass 0ASW (1508.18 mg/kg FM), which presented also a high C/N ratio (13.82). No significant differences were detected between frass 0ASW and 75ASW in terms of N and C/N ratio, while significant lower amounts of C and TOC, and higher amounts of S and P were measured in frass 75ASW than in 0ASW.

Discussion

Based on the authors' knowledge, this is the first study investigating the potential of BSF for reducing fresh ASW. Recently, Liland et al.¹⁰ and Schmitt et al.⁹ tested the use of ASW for rearing BSF, with focus on growth and chemical quality of insects, without considering the aspects related to the waste conversion process. Additionally, an oven drying process was applied to ASW in those studies. While drying might be needed to control substrate moisture more effectively¹², it is generally unfavourable in terms of economical and quality considerations, being responsible for vitamins degradation and microbial inactivation¹³. Considering that the present study utilized fresh ASW, such negative effects were not expected. Additionally, in the present study, ASW was collected daily, ensuring that it did not accumulated over time. According to Banks et al.¹⁴, accumulation of faeces may lead to anoxic conditions which might lead to reduced larval feeding and survival.

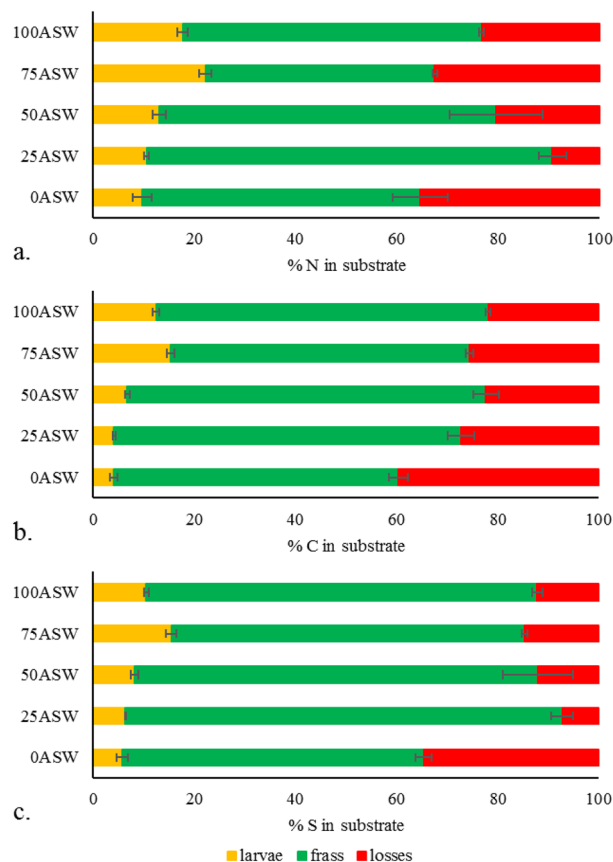


Figure 3. Distribution (%) of nitrogen (a), carbon (b) and sulphur (c) between larvae and frass as results from the mass balance approach. Error bars correspond to the standard error computed on 3 replicates/treatment. Losses represent the percentage of each element (nitrogen, carbon or sulphur) not recovered in larvae and frass (i.e. emitted as gas in the environment).

Parameter	0ASW	25ASW	50ASW	75ASW	100ASW
DM	29.90 ± 0.45 b	29.88 ± 1.48 b	32.82 ± 0.93 b	39.53 ± 0.35 a	25.32 ± 0.57 c
VS	87.60 ± 0.52 a	89.71 ± 2.15 a	88.20 ± 0.46 a	86.27 ± 0.36 a	80.62 ± 0.40 b
Protein	43.13 ± 0.82 b	48.52 ± 3.20 a	40.25 ± 1.02 b	33.90 ± 0.46 c	40.60 ± 0.60 b
Lipids	13.40 ± 0.52 ab	10.09 ± 1.53 b	16.80 ± 0.68 a	15.63 ± 0.91 a	2.11 ± 0.15 c
NFC	13.13 ± 1.65 b	11.65 ± 1.53 b	11.30 ± 0.85 b	20.34 ± 0.74 a	22.94 ± 0.56 a
NDF	17.94 ± 0.48 ab	19.45 ± 1.42 a	19.85 ± 1.08 a	16.40 ± 0.36 ab	14.97 ± 0.36 b
ADF	8.24 ± 0.09 a	8.35 ± 1.21 a	9.51 ± 0.36 a	8.12 ± 0.18 a	8.31 ± 0.29 a
ADL	1.75 ± 0.15 ab	1.25 ± 0.12 b	1.83 ± 0.41 ab	1.33 ± 0.25 ab	2.45 ± 0.18 a

Table 4. Chemical composition of BSF larvae reared on the different experimental substrates (mean ± Standard Error, n = 3). The values are in % of dry matter. ASW aquaculture solid waste, DM dry matter, VS volatile solids, NFC not fibrous carbohydrates, NDF neutral detergent fibres, ADF acid detergent fibres, ADL acid detergent lignin, C/N carbon to nitrogen ratio. Values within a row followed by different letters are significant different (P < 0.05).

Effect of different substrates on BSF performances

Results from this study showed that BSF larvae can effectively convert fresh ASW, seize nutrients from it and therefore reduce its polluting potential. Although treatment with pure ASW (100ASW) yielded the lowest substrate reduction, AF and insect growth, a comprehensive analysis of nutrient reduction and retention reveals a noteworthy assimilation by the BSF larvae. The observed low performances in the substrate 100ASW could be attributed to the feeding rate based on dry matter content. In the present experiment, a similar amount of fresh feed (300 g) was provided in each treatment. It resulted in a constant feed volume (substrate height ~ 2 cm) and larval density (0.6 larvae/cm²), two parameters considered crucial for the BSF larvae rearing¹⁵. As pure ASW

Parameter	0ASW	25ASW	50ASW	75ASW	100ASW
DM (% FM)	85.25 ± 1.01 a	85.79 ± 0.39 a	82.47 ± 1.77 a	83.55 ± 1.61 a	87.03 ± 0.14 a
VS	93.22 ± 0.45 a	91.57 ± 0.85 ab	89.76 ± 0.04 b	86.28 ± 0.27 c	72.70 ± 0.24 d
Lipids	0.73 ± 0.10 b	0.96 ± 0.14 b	1.95 ± 0.08 a	1.98 ± 0.20 a	0.70 ± 0.03 b
Hemicellulose	8.72 ± 0.38 b	7.84 ± 0.07 c	6.34 ± 0.07 d	7.68 ± 0.08 c	15.88 ± 0.10 a
Cellulose	4.72 ± 0.56 c	4.88 ± 0.12 c	5.22 ± 0.17 c	6.94 ± 0.08 b	11.58 ± 0.05 a
Lignin	1.51 ± 0.18 c	2.12 ± 0.16 bc	2.05 ± 0.09 bc	2.85 ± 0.18 b	8.23 ± 0.36 a
Nitrogen	3.29 ± 0.21 c	3.91 ± 0.02 b	3.36 ± 0.31 c	3.08 ± 0.10 c	4.85 ± 0.02 a
Carbon	45.06 ± 0.02 a	44.49 ± 0.17 a	44.28 ± 0.17 ab	43.27 ± 0.82 b	38.60 ± 0.06 c
TOC	40.24 ± 0.21 a	39.52 ± 0.73 a	40.00 ± 0.40 a	37.30 ± 0.85 c	34.23 ± 0.09 d
Sulphur	0.32 ± 0.02 c	0.36 ± 0.00 c	0.37 ± 0.01 c	0.46 ± 0.01 b	0.86 ± 0.00 a
C/N	13.82 ± 0.92 a	11.37 ± 0.11 b	13.43 ± 1.27 ab	14.06 ± 0.17 a	7.96 ± 0.03 c
NH ₄ (mg/kg FM)	1508.18 ± 149.64 c	2035.26 ± 229.27 c	3287.58 ± 199.03 b	5003.94 ± 48.71 a	3243.12 ± 104.28 b
NO ₃ (mg/kg FM)	1.13 ± 0.30 a	2.00 ± 0.16 a	3.05 ± 1.76 a	0.68 ± 0.09 a	0.85 ± 0.02 a
P (g/kg FM)	5.33 ± 0.55 d	6.65 ± 0.23cd	7.37 ± 0.30 c	11.51 ± 0.24 b	25.62 ± 0.17 a
K (g/kg FM)	13.00 ± 1.72 a	12.81 ± 0.65 a	12.66 ± 0.18 a	11.18 ± 0.47 a	0.90 ± 0.03 b
pH	7.03 ± 0.19 ab	6.62 ± 0.13 b	7.22 ± 0.22 ab	7.23 ± 0.06 ab	7.33 ± 0.02 a

Table 5. Chemical composition and pH of frass generated by BSF larvae reared on the different substrates (mean ± standard error, n = 3). The values are in % of dry matter, unless otherwise specified. ASW aquaculture solid waste, DM dry matter, FM fresh matter, VS volatile solids, TOC total organic carbon, C total carbon, N total nitrogen, NH₄ ammonium nitrogen, NO₃ nitric nitrogen, P elemental phosphorous, K elemental potassium. Values within a row followed by different letters are significant different (P < 0.05).

presented a very limited DM ($7.95 \pm 0.06\%$), the amount of energy and nutrients provided to the larvae in this treatment was low and might not have been sufficient for supporting larval growth. Diener et al.¹⁶ and Manurung et al.¹⁷ have also reported lower larval weight and longer development time when limited feed was offered. However, despite the extremely limited amount of dry substrate, high larval survival was recorded, suggesting that the amount of provided diet was still enough for reaching the critical weight and supporting larval development¹⁸. It might be due to the high amount of nitrogen and protein content of the ASW. It is well known that nitrogen is crucial for building the body structure and reaching the larval critical weight^{19–21}. As protein concentration of larvae growth on treatment 100ASW was similar to the larvae 0ASW, it can be stated that sufficient amount of protein was provided to the larvae in this treatment. When the nitrogen requirement is fulfilled, BSF larvae start accumulating lipids as energy reserve (fattening), resulting in higher final weight^{22,23}. The low lipid concentration in larvae 100ASW suggests that very limited amount of energy was accumulated in these larvae. As a balanced C/N ratio and nutrients level are responsible for reaching high larval weight^{24,25}, increasing the energy level of the feed should be accompanied by a proportional increase of the nitrogen content. However, when the critical weight is reached, the consumption of nitrogen and carbon is not balanced, resulting in lower larval nutrient retention and therefore higher amount of nutrients volatilization^{26,27}. Recent research on earthworms confirms such findings²⁸, suggesting that low feeding rate in waste management should be pursued whether the aim is to reduce the overall environmental impact of the system.

The high nutrients retention and reduction observed in 100ASW were not accompanied by a similarly high dry substrate reduction. It suggested that despite the low feeding rate, the amount of substrate used by BSF larvae was very low. The main reason for it could be found on the high level of indigestible fibres (i.e. cellulose and lignin) detected in substrate 100ASW²⁹. Similar results were obtained by Rehman et al.³⁰ for BSF larvae raised on pure cow manure. Indeed, although such fibres can be degraded by microorganisms living in the gut of BSF larvae³¹, very limited hemicellulose and cellulose degradation and total absence of lignin degradation were noticed in treatment 100ASW. Addition of CF to the ASW led to an increasing concentration of carbohydrates and total VS, while percentage of fibres decreased. Accordingly, C/N ratio as well as carbohydrate to protein ratio increased reaching values of 10.78–13.33 and 3.01–3.49, respectively. While reduction of fibre content in substrate was beneficial for promoting direct larval feed consume^{32–34}, more balanced nutrient composition could favour a better microbial degradation of the material that cannot directly be digested by BSF larvae²⁴. It followed that the substrate with lower fibre content and the most balanced C/N ratio, i.e. 0ASW, was the substrate better used by the BSF larvae, explaining the significantly higher substrate reduction and AF observed in this treatment. Nevertheless, the high FCR (i.e. amount of feed needed for increasing the larvae weight of 1 g) and low BCR (i.e. amount of feed converted in larval biomass) computed for treatment 0ASW indicated that BSF larvae did not efficiently consumed this substrate. Suitable explanation could be found in the scarce homogeneity of the substrate³⁵. As CF used in our experiment presented high dry matter ($90.16 \pm 0.16\%$), a significant amount of water was added. It resulted in an observable water phase on top of the substrate that might have been hindered oxygen dissolution, resulting in an anaerobic environment responsible for low larval activities^{12,36}. Increasing the amount of ASW in substrates resulted in a reduction of extra water added, as the ASW supplied most of the moisture in these substrates. As consequence, an improvement on the substrate structure could be assumed, explaining the better substrate to biomass conversion (lower FCR and higher BCR) detected in substrates 25ASW, 50ASW and 75ASW. However, unlike to our expectation, no clear trend was observed between ASW in diet

and larvae performances. The only treatment that resulted significantly better was indeed the treatment 75ASW, which gave the heaviest larvae. Compared with 0ASW, 25ASW and 50ASW diets, the 75ASW substrate showed higher lipids and lower VS, protein and NFC. The contents of N and $\text{NH}_4\text{-N}$ were significantly higher, while the C/N ratio was lower. Altogether, such conditions resulted in a significant lower FCR and higher BCR which were comparable to pure poultry manure or mixtures of dairy and chicken manures³⁷. It suggests that reduction in carbohydrate/protein ratio as well as C/N ratio of the substrate might be an interesting strategy for improving BSF larvae production¹⁹, although higher C and N losses and, therefore, higher environmental impact of the process should be considered^{38,39}.

Effect of different substrates on BSF larvae nutritional composition

Although the aim of this study was to investigate the ability of BSF larvae in growing on ASW based substrate and reducing the environmental impact of the aquaculture sector, an important aspect that should always be considered is the quality of the reared insects. Different studies have shown that quality of harvested insects is strictly dependent on the nutritional composition of the diet used as rearing substrate³⁷. However, different nutrients show different patterns, with crude protein being more stable than lipids and ash⁴⁰. In the present study, protein content of larvae varied from 33 to 50% (DM basis). The lowest protein content in 75ASW could be attributed to relative higher carbohydrates and lipids proportion, as well as the possible proximity to the pupation stage⁴¹. Specifically, the insects were harvested after observing the first pre-pupae. It means that a large number of larvae could not have yet reached the pre-pupal stage, partially hindering the results⁴². Lipids, which are accumulated as energy reservoir in the insect fat body, were constant, although significant different, between all the treatments, except for larvae 100ASW, where only 2% of lipids were detected. As already stated, feeding rate in this treatment was not enough for supporting BSF fattening, explaining the extremely low amount of fat accumulated. Interestingly, although larvae 75ASW were significantly heavier than the others, they did not show the highest lipid content. However, the same larvae displayed high NFC level. Since lipids can be synthesized from carbohydrates, it was possible that higher lipid concentration might be reached in a later stage. Ash content strictly followed the trend observed in the diets. It is consistent with previous findings^{9,10,41} and might be explained by the presence of residual feed in the larval gastro-intestinal tract¹³.

Effect of different substrates on frass quality

Frass are the secondary material generated by insect-based waste management system. They consist of a nutrient rich material that, whether not correctly handled, may results in an important source of environmental impact^{43,44}. Current practices include use of the frass as soil conditioner/fertilizer in agriculture. Several studies have focused on the nutritional composition of this material, evaluating its main effects on plant growth and soil health^{8,45}. Frass obtained in this study showed high content in nitrogen and potassium, with NPK ratio ranged between 5:1:1 (75ASW) and 6.5:1:2.5 (0ASW). The only exception was represented by frass generated from treatment 100ASW, which showed a significantly higher content in phosphorous, with NPK ratio of 35:28.5:1. These results confirm previous findings reviewed by Basri et al.⁴⁵, which showed that diets richer in protein generated frass richer in nitrogen, while diets richer in carbohydrates produces frass with low phosphorous concentration. As all the substrates used in the present study were rich in proteins, nitrogen-rich frass could be expected. On the other hand, as all the substrate except 100ASW were rich in carbohydrates, it is reasonable that the only frass with high amount of phosphorous was the one from treatment 100ASW. Additionally, it is interesting to notice that absolute amount of these nutrients followed a clear trend among treatments with substrates richer in ASW producing frass richer in nitrogen and phosphorous and poorer in potassium. However, no similar trends were observed in the initial substrates. As phosphorous and potassium are more stable than nitrogen in the substrates²⁹, it can be speculated that assimilation of such nutrients by BSF larvae depends on the physiological status of the larvae, resulting in different concentrations in the obtained frass. Concerning the nitrogen content, a distinction between ammonium nitrogen and nitric nitrogen should always be done. Regardless of the treatment, frass obtained in our study appeared as an important source of ammonium nitrogen. Such nitrogen form is known for being more stable within the soil, being less available for plant absorption and atmospheric volatilization⁴⁶. Although higher level of ammonium nitrogen over nitric nitrogen can be preferred in terms of environmental impact reduction, the same condition indicates a low maturity of the frass⁴⁵, which should go through a further composting process before being applied in agriculture⁴⁷. This condition has been frequently observed in BSF mediated bioconversion processes and it is usually associated to the short rearing period⁴⁸. As in the present study, the longest residence time was only 10 days, it is reasonable to think that frass were far from being mature for agriculture application. Low compost maturity can also be assumed by considering the C/N ratio. Such ratio, although in line with the values reported in literature for BSF frass⁴⁵, was particularly low and not significantly different from the C/N ratio of the initial substrates. As stable compost should present a C/N ratio between 20 and 40 for not being toxic for plants⁴⁹, and significant increase of C/N ratio from substrate to frass was only observed in treatment 75ASW (from 10.78 to 14.06), it can be concluded that a further composting process is warranted before they can be used in agriculture.

Conclusion and future research direction

The present work clearly shows that fresh ASW can be used for BSF larvae rearing, combining biomass production and nutrients recovery with waste management and pollution reduction. Although the limited feeding ratio applied on treatment 100ASW did not allow us to draft definitive conclusions, the high larval survival recorded on such treatment, combined with a larval protein content similar to the other treatments, led to conclude that pure fresh ASW is potentially suitable for rearing BSF larvae. Additionally, the feed limitation of the aforementioned treatment suggests a better resource utilization by BSF larvae, resulting in lower nutrients loss

and FCR, and higher amount of nutrients retention. Addition of chicken feed to the ASW led to reduction of ash and fibres, while easy to digest carbohydrates and C/N ratio increased. These conditions resulted in better larval growth, bioconversion ratio, feed assimilation and substrate reduction. However, the efficiency on using the available resources decreased as lower larval nutrients retention and higher nutrients loss were observed. The treatment 75ASW was capable of most effectively combining ASW reduction with larval biomass production and low environmental impact. Frass generated from the different treatments showed high content in nitrogen and potassium, while phosphorous was relevant only in frass from treatment 100ASW. The C/N ratio of frass was similar to the C/N ratio of substrates, suggesting that a further composting period was needed before their application in agriculture.

Future research should aim to evaluate whether a higher feeding rate can improve the larval growth on pure fresh ASW, as well as to explore alternative materials other than CF for improving and optimizing the insect-based aquaculture waste management process. Furthermore, the microbial and chemical safety of insects produced on ASW, as well as the effect of BSF frass as a fertilizer and different post-composting processes for their stabilization should be considered in order to promote the full utilization of BSF in the circular economy. Finally, the ability of BSF larvae to reduce the amount of nutrients lost in the environment by decreasing their feeding rate without negatively affecting the overall performance of the system deserves more attention.

Methods

Insects

BSF eggs laid on corrugated cardboard within 36 h were purchased from Hermetia Baruth GmbH (Baruth/Mark, Germany) and placed in 2 Litres 21 × 15 × 11 cm polypropylene transparent boxes (Ikea Deutschland GmbH & Co. KG, art. 603.591.52, Munich, Germany) for hatching. The boxes were placed inside a climate chamber set at 30 ± 1 °C, $70 \pm 5\%$ RH and photoperiod 0:24 (L:D). Young larvae were carefully handled with a camel brush and transferred in similar polypropylene box filled with chicken feed (CF) (all-mash A Mehl mit Cocc., Deuka companion GmbH & Co. KG, Dusseldorf, Germany) moistened with distilled water at 50% of moisture and housed in a climate chamber at 28 ± 1 °C, $65 \pm 5\%$ RH, 0:24 (L:D). Eggs started hatching approximately 24 h after their purchase, but only the larvae hatched after further 12 h (from 36 to 48 h from the purchase) were used in the experiment.

Aquaculture waste

Aquaculture discharged water from pikeperch (*Sander lucioperca*) was provided by the Institute of Inland Fisheries (Potsdam-Sacrow, Germany). Six-month old fishes ($n=940$, average body weight: 103 g) were reared in a semi-industrial scale RAS equipped with 6 polyethylene rearing tanks (volume 1.3 m³ each), a moving bed biofilm reactor (biofilm surface 1000 m²), automatic feeders (Pflanzer Fütterungssysteme, Germany) and a drum filter (Hydrotech HDF 801 1G, Sweden). The water was kept at constant temperature of 23 °C and pH of 6.8 and it was surface-illuminated 16 h/day with dimmed light of 5 lx. Fishes were fed with commercial aquafeed (Biomar Elico Sigma 870F 3 mm, Denmark) every 4 h during the illumination period. Daily feeding rate was set at 1.5% of the fish biomass, correspondent to 1.4–1.7 kg of feed per day. For each kg of administered feed, 380 L of make-up water were added.

Approximately 50 L of drum filter wastewater containing c.a. 0.02% of solids (fish faeces and feed residuals) were daily collected for 2 weeks (from Monday to Friday, total ~ 500 L). In order to reduce the water level, daily collected wastewater was submitted to concentration, consisting in sedimentation (1 h, room temperature), followed by filtration ($\varnothing$ 0.2 mm) and centrifugation (10 min, 3900 rpm, 20 °C). The separated water was discarded and the resulting sludge (ASW, moisture $90.93 \pm 0.26\%$) was kept frozen at -18 °C until the beginning of the rearing experiment.

Experimental set-up

Frozen ASW was thawed at room temperature and thoroughly hand-mixed. Based on a preliminary experiment, five experimental substrates consisting of CF (all-mash A Mehl mit Cocc., Deuka companion GmbH & Co. KG, Dusseldorf, Germany) supplemented with ASW in different percentages (0—control, 25, 50, 75 and 100%) were formulated by hand-mixing the two ingredients. Substrate moisture was adjusted at 70% (except 100% ASW) by adding distilled water when needed (Table 1). This moisture level was chosen based on the data published by Bekker et al.¹², who observed better BSF larvae growth with moisture ranged between 65 and 75%.

For each treatment, three experimental boxes (three replicates/substrate) were filled with 300 g (wet weight) of substrate (bulk volume ~ 450 cm³) and exactly 200 5-day old larvae (larval density 0.6 larvae/cm²). The amount of diet and the larval density were chosen for avoiding anaerobic condition which may impact larval development and substrate reduction¹⁵. It resulted in a significantly lower amount of dry material provided in substrate 100ASW.

Experimental boxes consisted of 2 L 21 × 15 × 11 cm polypropylene transparent box covered with a polypropylene transparent lid (Ikea Deutschland GmbH & Co. KG, art. 503.617.92, Munich, Germany) equipped with a rectangular hole (15 × 10.5 cm) covered with polyester black mosquito net (1 × 1 mm mesh, HaGa-Welt GmbH & Co. KG, Nordstemmen, Germany). These boxes were placed in a climate chamber set at 27 ± 1 °C, $70 \pm 5\%$ RH, 0:24 (L:D). For each single box, rearing was stopped and larvae were harvested when the first pre-pupae was observed in that box. It resulted in different harvesting time for each box (within the same treatment). At harvesting, all pre-pupae and larvae from the selected box were handpicked with forceps, washed with distilled water, dried with paper towel and inactivated by freezing at -20 °C for 1 h. Larvae/pre-pupae, frass and samples of initial substrates were kept frozen at -30 °C until the chemical analysis.

Growth and waste bioconversion performances

In order to evaluate the ability of BSF larvae to grow on pure or CF-supplemented ASW, 10 larvae were randomly sampled every second experimental day (day 0, 2, 4, 6, 8, 10), cleaned with towel paper and weighted (Sartorius CPA224S-0CE Analytical Balance, Sartorius AG, Goettingen, Germany).

Total larval biomass, larvae numbers as well as diet and frass weights were recorded at the beginning and at the end of the rearing period. In order to better understand the changes occurring on the material during the bioconversion process, pH of diets and frass were measured³⁰. Briefly, 1 g of material was mixed with 10 g of distilled water (1:10 w/w), the mixtures was vortexed for 5 s, incubated for 1 h at room temperature and the pH was measured with a benchtop pH meter (SI Analytics Lab 850 pH-Meter, SI Analytics GmbH, Mainz, Germany).

Bioconversion and growth performances were evaluated by computing the following indexes^{37,42}:

- (1) Larval survival (%) = (final larval number/initial larval number) × 100.
- (2) Development time (day) = larvae harvesting day – larvae seeding day.
- (3) Gained weight per larvae (GW; mg) = final larval fresh weight – initial larval fresh weight.
- (4) Assimilated feed (AF; g) = total substrate dry weight – total frass dry weight.
- (5) Feed conversion ratio (FCR) = (dry weight of ingested feed)/(final larvae dry weight – initial larvae dry weight).
- (6) Biomass conversion rate (BCR; %) = [(total final larvae fresh weight – total initial larvae fresh weight)/total substrate fresh weight] × 100.
- (7) Substrate reduction (%) = [(total substrate dry weight – total frass dry weight)/total substrate dry weight] × 100.

Chemical analysis

Initial substrate, residual frass and pre-pupae were analysed for dry matter (DM), ash, total nitrogen (N), total carbon (C), total sulphur (S), crude lipids, neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL). Furthermore, total organic carbon (TOC), elemental phosphorous (P), elemental potassium (K), contents of ammonium (NH₄-N), nitrite (NO₂-N) and nitrate (NO₃-N) were quantified in substrates and frass.

DM was determined by oven drying at 105 °C overnight (NFTA 2.1.4 official moisture method). Lipids were quantified with the Soxhlet method, by using petroleum ether as extraction solvent (AOAC, 2000—method 960.30). N was quantified with the Kjeldahl method (AOAC, 2000—method 992.15) and crude protein (CP) were computed by applying the factors 5.70 for CF⁵⁰, 3.11 for ASW⁵¹ and 4.76 for BSF larvae⁵². NDF and ADF were quantified by gravimetric methods through digestion of the milled sample at 100 °C with neutral detergent or acid detergent solution respectively, followed by oven drying for 3 h at 102 °C. ADL was determined after further digestion for 3 h in 70% H₂SO₄ and oven drying (2 h, 105 °C). Hemicellulose was computed as difference between NDF and ADF, while cellulose was calculated as ADF-ADL⁵³. Ash content was quantified through incineration of the sample at 550 °C for 4 h (DIN 38414-EN 12879), and total volatile solids (VS) were calculated by difference between DM and ash.

C and S were analysed through a catalytic raw combustion of the homogenised sample, operated under oxygen supply by the elemental analyser VARIO EL (Elementar Analysensysteme GmbH, Langenselbold, Germany), while P and K were quantified through atomic absorption spectroscopy (iCE 3300 AAS, Thermo Fisher Scientific, Waltham, USA) performed on the microwave acid digested sample (VDLUF method, volume 3-chapter 10). TOC was gravimetrically determined, evaluating the reduction of the acidic treated sample weighted after evaporation of CO₂ at 1200 °C (DIN-EN 13137:2001-12). Finally, NH₄-N, NO₂-N and NO₃-N were analysed with the ion chromatography system Dionex ICS-1000 (Thermo Fisher Scientific, Waltham, USA), equipped with IonPac AS9-HC (4 × 250 mm) separation column (Thermo Fisher Scientific, Waltham, USA) and ASRS-Ultra detector (Thermo Fisher Scientific, Waltham, USA) for anions (NO₃⁻, NO₂⁻) or IonPac CS16 (5 × 250 mm) separation column and CSRS-Ultra detector (Thermo Fisher Scientific, Waltham, USA) for cations (NH₄⁺).

Additionally, not fibrous carbohydrates (NFC) were determined for insects and substrates by applying the following formula²⁶:

- (8) $NFC = 100 - (\text{crude proteins} + \text{crude lipids} + \text{NDF} + \text{ash}).$

Chemical analyses were performed on oven-dried samples (60 °C, 48 h). Substrates were analysed in triplicate, while chemical analyses of larvae and frass were conducted in singlet.

Nutrients retention of larvae was evaluated by computing the following index for N, C and S:

- (9) $\text{Nutrient retention (\%)} = [(\% \text{Nutrient in larvae} \times \text{DM larvae}) / (\% \text{Nutrient in substrate} \times \text{DM substrate})] \times 100.$

A mass balance approach was applied for evaluating the overall conversion efficiency of the process.

Statistical analysis

Statistical analyses were conducted in R software version 4.1.0⁵⁴. Chemical composition, nutrient reduction and nutrient retention indexes were subjected to one-way analysis of variance (ANOVA). Normality and homoscedasticity of models residuals were evaluated by Shapiro–Wilk test and Levene test (package “car”) respectively. Beta regression (link logit) was applied when absence of normality was detected.

One-way analysis of covariance (ANCOVA) with development time as covariate, was used for comparing growth and bioconversion performances. Significance level was always set at 0.05 and Tukey HSD test for multiple comparison (package “multcomp”) was applied when significant differences were detected.

Larvae survival was analysed through general linear model (package “emmeans”) with binomial distribution (link logit), while general linear model with gamma distribution (link inverse) was used for modelling the development time. In both cases, Dunnett’s contrasts test (package “multcomp”) at significance level of 0.05 was used for computing the differences among treatments.

Generalised linear mixed model (GLMM, package “lme4”) with gamma-log link and substrate replicates as random effect (observation nested within the same box) followed by pairwise Tukey test with a significance level of 0.05 was used for evaluating the larvae growth over time^{55,56}.

Ethics approval and consent to participate

This study was approved by the regional food and feed safety authorities “327 Bereich Veterinär- und Lebensmittelüberwachung der Stadtverwaltung der Landeshauptstadt Potsdam, Geschäftsbereich Ordnung, Sicherheit, Soziales und Gesundheit, Fachbereich Ordnung, Sicherheit und Gesundheit” Brandenburg, Germany. The fish rearing was conducted by the Institut für Binnenfischerei e.V. Potsdam-Sacrow, authorisation number 386-1-384 issued by the Fachbereich Ordnung, Sicherheit und Gesundheit, Bereich Veterinär- und Lebensmittelüberwachung—Municipality of Potsdam.

Compliance with relevant guidelines and regulations

Animal housing, feeding experiment, sampling, killing operations, samples processing, animal waste management and analyses were carried out in accordance with the Deutsche Forschungsgemeinschaft (DFG) relevant guidelines and the Federal regulations (Article 20a of the Basic Law for the Federal Republic of Germany).

Compliance with ARRIVE guidelines

The current study was carried out in compliance with the ARRIVE guidelines when relevant methods were applied.

Data availability

All data generated or analysed during this study are included in this published article.

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Author contributions

G.R.: conceptualization, data acquisition and analyses, writing-reviewing and editing. S.O.: conceptualization, methodology, writing-reviewing and editing. A.M.-B.: writing-reviewing and editing, resources. O.K.S.: writing-reviewing and editing, funding acquisition, supervision. All authors have read and agreed to the final draft of the manuscript.

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Competing interests

The authors declare no competing interests.

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A9. Insect-mediated valorisation of anaerobically digested aquaculture waste: bioconversion performances, nutritional composition and microbial safety of black soldier fly larvae

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ARTICLE

Insects-mediated valorisation of anaerobically digested aquaculture waste: bioconversion performances, nutritional composition and microbial safety of black soldier fly larvae

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Recent studies suggested that fresh aquaculture waste (ASW) could satisfactorily be treated by black soldier fly larvae. However, pre-treatments such as drying or dewatering, which significantly modified the chemical, physical and microbiological properties of the waste, were always applied. On the other hand, industrially-generated aquaculture waste might be bulk-accumulated for long time and not always be suitable for pre-treatments. Therefore, the present study aimed to evaluate the ability of BSFL on converting bulk-accumulated ASW from aquaculture industrial facility, while generating high quality and safe insects. Five substrates, consisting of different mixtures of ASW (0, 25, 50, 75, 100%) and chicken feed were prepared and offered to the larvae (feeding ratio = 0.46 g-DM/larvae). Increasing amounts of ASW in diet resulted in progressive lower larval growth, with the substrate consisting of 100% ASW not allowing any growth. Larvae raised on substrates containing high amount of ASW showed decreased concentration of lipids and protein, while the amount of ash increased. Amino acid composition and fatty acid profile of BSFL appeared to be stable. High microbial contamination was always detected in the reared larvae, although no pathogens were found in any insect. It was concluded that BSFL could be used for managing anaerobically digested ASW, although an amending material able to improve the quality of the substrate is needed. Obtained insects might be valorised as feed or employed for extracting nutritional components to add to food, veterinary, pharmaceutical or agricultural products.

Introduction

As the importance of aquaculture production is rising¹, an increase in the production of aquaculture solid waste (a.k.a. aquaculture sludge; ASW) is inevitable. Such waste consists of uneaten feed and fish faeces², and is produced after concentration processes are applied to the aquaculture wastewater³. Being rich in nitrogen, phosphorous

and organic matter, ASW may represent a threat for aquatic organisms and the environment⁴. Traditional systems of aquaculture waste management include landfill discharge, incineration, wetland and phytoremediation, aerobic and anaerobic composting, fermentation, chemical or enzymatic hydrolysis and fertilization^{5,6}. Although characterized by high efficiency, these methods are associated with high capital or operating costs and/or generation of undesirable by-products⁵. Modern technologies combining aquaculture with other organisms including plants and microorganisms have also been developed. Such methods, referred as integrated multi-trophic aquaculture systems, aim to associate nutrients sequestration and waste management with biomass production⁵. Based on such concept, the idea of using insects and heartworms for ASW management has been proposed^{7,8}.

Several insect species have been described as capable of growing on decaying materials such as waste and manure⁹. Among those, black soldier fly (*Hermetia illucens*) larvae (BSFL) were identified as the most promising species¹⁰. Recent studies have shown that ASW may be utilized by these larvae, although pure aquaculture waste did not result in satisfactory growth performances^{7,11}. The physical state as well as the nutritional composition of the waste have been suggested as crucial variables¹², with fresh ASW showing better insect growth than oven dried ASW¹³.

ASW originated from a pilot-scale RAS (recirculating aquaculture system) unit was used in a recent study by Rossi et al. (2023)¹³. Since ASW was collected daily, it did not accumulate in sedimentation ponds, suggesting that its physio-chemical properties were not comparable to the ASW produced at industrial scale, where bulk

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Supplementary Information available:

Supplementary S1 A. Chemical composition (% dry matter, average $\pm$ standard error, n=3) of the raw material used in the experiment.

Supplementary S1 B. Fatty acid profile (% total fatty acid, average $\pm$ standard error, n=3) of the raw material used in the experiment.

Supplementary S2. Regression analyses between ASW weight in initial diet (g) and final larval weight (mg).

Supplementary S3 A. Correlation analyses between larval growth and bioconversion performances and chemical quality of the substrates.

Supplementary S3 B. Correlation analyses between larval proximate composition and chemical quality of the substrates.

Supplementary S3 C. Correlation analyses between larval fatty acid and fatty acid profile of the substrates.

Supplementary S3 D. Correlation analyses between larval amino acids and amino acid profile of the substrates.

Supplementary S4. Microbial confirmation results from MALDI-ToF analyses

accumulation is expected^{14,15}. Therefore, to promote the use of BSFL for large-scale ASW management, further research is needed. Therefore, the first aim of this study was to evaluate the ability of BSFL to utilize anaerobically digested ASW in their feed.

Additionally, while insect biomass production should always be considered when insect-mediated bioconversion is investigated, other variables such as nutrient recovery efficiency, microbial safety and chemical quality of the final insect biomass should also be considered. Such aspects are of primary importance as a fully transition to the circular economy can only be ensured if a satisfactory use of the end-products is implemented¹⁰. Generated insect biomass might be used for feeding animals as well for extracting single components to be used as raw material for energy or drugs production¹⁶⁻¹⁸. Therefore, the second aim of this study was to evaluate the chemical quality as well as the microbiological safety of the generated insects.

Materials and Methods

Raw material

ASW produced by an industrial inland aquaculture facility located at the Blankensee lake (Trebbin, Germany), consisting of a mixture of European catfish (*Silurus glanis*) and pikeperch (*Sander lucioperca*) manure were used in this experiment. According with the farm layout, aquaculture waste, generated from 10 breeding tanks (overall volume 84 m³) hosting approximately 500 animals (average age 1 year, average weight 300 g for pikeperch and 3 Kg for catfish), were collected in conical compartments and mechanically drained to a settling tank. Excess of water was removed and dewatered waste were collected in an anaerobic sedimentation pond (overall volume 4 m³). Approximately 50 Kg of six months old ASW were collected and submitted to a concentration process consisting in sedimentation (1 hour, room temperature) and filtration (Ø 0.2 mm). Final material was frozen at -18°C until further use.

Black soldier fly eggs laid on corrugated cardboard were purchased from Hermetia Baruth GmbH (Baruth/Mark, Germany) and placed in a climate chamber at 30 ± 1°C and 70 ± 5% RH for hatching. Neonates larvae were transferred into a 2 L polypropylene transparent box (Ikea Deutschland GmbH & Co.KG, Munich, Germany) half filled with commercial chicken feed (Deuka Companion GmbH & Co. KG, Dusseldorf, Germany) moistened with distilled water at 50% of final moisture. Boxes were housed in a climate chamber set at 28 ± 1°C, 65 ± 5% RH and 0:24 (L:D). Larvae were let develop for 6 days before the beginning of the experiment.

Experimental set-up

Frozen ASW were thawed overnight at room temperature and hand-mixed. Following the experimental set-up reported in Rossi et al. (2023)¹³, five substrates consisting of different percentage (0, 25, 50, 75, 100%) of ASW (dry matter = 25.01 ± 1.75%) and chicken feed (dry matter = 89.98 ± 0.19%) were prepared (table 1). Distilled water was added when needed with the aim of reaching a final substrate dry matter of approximately 30%. For every mixture, correct amount of each ingredient (ASW, chicken feed, distilled water) was calculated

by considering a final substrate mass of 300 g. The computed masses were therefore directly added into a 2 L polypropylene rearing box, and thoroughly hand mixed. As pure ASW (substrate 100ASW) showed a final moisture content higher than 70%, a surplus of material was added. It allowed to ensure the same feeding ratio in all the treatment (0.45 g-dry substrate/larvae), avoiding that the different total solid content could hinder the results.

For each treatment, six boxes were prepared. Three boxes were used for evaluating growth, bioconversion performances and chemical quality of insects (3 biological replicates), while the other three were intended for microbial investigation (3 biological replicates). After preparation, each substrate was left stabilize for 24 hours at 25°C prior of adding the larvae. Such operation was needed to allow the free water to be absorbed by the dry components of the medium, resulting in a homogeneous substrate. After this period, two-hundred of 6-days old larvae were added on top of each substrate and the boxes were closed with a polypropylene transparent lid (Ikea Deutschland GmbH & Co.KG, Munich, Germany) equipped with a rectangular 15x10.5 cm hole screened by polyester black mosquito 1x1 mm net (HaGa-Welt GmbH & Co. KG, Nordstemmen, Germany). All the boxes were transferred into a HGC 1514 V Simpac Fitotron (Vötsch Industrietechnik, Balingen-Frommern, Germany) chamber set at 27 ± 1°C, 70 ± 5% RH, 0:24 (L:D) and let develop for 12 days, i.e. up to the appearance of the first prepupae in all the substrate:

Evolution of growth and bioconversion performances

In order to understand the suitability of anaerobically digested ASW to be used as rearing substrate for BSFL, exact weights of initial substrate, starting larvae, frass and mature larvae were recorded. Additionally, numbers of seeded and harvested larvae were also documented. Larvae performances were evaluated through the following equations (Rossi et al., 2023)¹³:

$$\text{Larval survival (\%)} = (nLf/nLi) \times 100$$

$$\text{Assimilated feed (g)} = dWs - dWf$$

$$\text{Feed conversion ratio (FCR)} = \text{Assimilated feed}/(dWLf - dWLi)$$

$$\text{Bioconversion rate (\%, BCR)} = ((WLf - WLi)/Ws) \times 100$$

$$\text{Substrate reduction (\%)} = ((dWs - dWf)/dWs) \times 100$$

where *nLf* and *nLi* were the final and the initial larval number respectively, *WLf*, *WLi* and *Ws* indicated the final larvae fresh weight, initial larvae fresh weight and substrate fresh weight, while *dWLf*, *dWLi*, *dWs* and *dWf* stood for final larval dry weight, initial larvae dry weight, substrate dry weight and frass dry weight.

Larval growth on the different substrates was evaluated during the rearing experiment by sampling 10-larvae every second experimental day (day 2, 4, 6, 8, 10, 12). Such larvae were randomly picked up with forceps, cleaned with towel paper and weighted on an analytical scale (Sartorius AG, Goettingen, Germany), prior to be returned to the respective substrate.

Table 1. Composition and dry matter (% of fresh matter) of the experimental substrates.

Treatment	ASW ¹ (%)	CF ² (%)	ASW (g)	CF (g)	Water (g)	Total weight (g)	Dry matter ³ (%)
0ASW	0	100	0.00	100.09	199.91	300.00	32.91 ± 1.36 a
25ASW	25	75	30.79	92.38	176.83	300.00	33.20 ± 0.86 a
50ASW	50	50	80.05	80.05	139.90	300.00	29.49 ± 0.77 ab
75ASW	75	25	171.48	57.16	71.36	300.00	29.97 ± 0.18 ab
100ASW	100	0	360.00	0.00	0.00	360.00	25.56 ± 1.89 b

¹ ASW, aquaculture solid waste² CF, chicken feed³ Dry matter is expressed as mean ± standard error of 3 independent repetitions

Chemical analysis

Proximate analyses, including dry matter (DM), ash, total lipids, neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), total carbon (C), total nitrogen (N) and total sulphur (S) of substrates and insects were performed according to Rossi et al. (2023)¹³. Gross energy was quantified through the bomb calorimetry method as reported in Hopper et al. (2023)¹⁹. Hemicellulose, cellulose and lignin contents were calculated according to Peguero et al. (2023)²⁰. Amount of not fibrous carbohydrates (NFC) were calculated as difference between 100 and sum of crude protein, total lipids, NDF and ash.

Fatty acid profile of substrates and larvae was determined according to Lepage and Roy (1986)²¹ with modifications. Specifically, a defined amount of analytical material was placed in a glass tube and a methanol-hexane mixture was added. Subsequently, acetyl chloride was added and the sample was heated, followed by the addition of potassium carbonate solution. After centrifugation, the measurement was carried out by gas-chromatography (GC TRACE 1300, Thermo Scientific, Dreieich, Germany).

Amino acid profile was determined by ion-exchange chromatography (biochrom 30+ Amino Acid Analyzer, Laborservice Onken GmbH, Gründau, Germany) after hydrolysis. In case of methionine and cysteine, sample hydrolysis was preceded by oxidation. Tryptophan was not determined.

All the chemical analyses were carried out in duplicate, and the results were mediated. As treatment 100ASW did not result in satisfactory insect growth, BSFL originated from this treatment were not analysed.

Evaluation of microbial safety

The three boxes for each treatment intended for microbial analyses were prepared by following the same procedure as in section 2.2, but ensuring the highest hygienic standards. Briefly, prior of adding substrate and larvae, experimental boxes and utensils were carefully cleaned and disinfected with 70% ethanol. After disinfection, approximately 310 g (300 g for rearing + 10 g for analyses) of each substrate were prepared and left stabilize for 24 hours. Before adding insects, approximately 10 g of substrate from each box were sampled with sterile spoons, placed in a Nasco Whirl-Pak sterile bag and frozen at -80°C for further microbiological analysis. The remaining 300 g were seeded with 200 BSFL and incubated for 12 days.

After this period, approximately 10 g of mature larvae/prepupae were randomly picked up with sterilized forceps, washed in autoclaved distilled water, dried with autoclaved tissue and transferred into a Nasco Whirl-Pak sterile bag.

For microbial analyses, 3 g of material and 27 g of autoclaved buffered peptone water were weighted in a sterile stomacher bag and homogenised at speed 4 for 2 min. The homogenate was serially diluted with sterile buffered peptone water and plated on selective media, according to the ISO standards. Aerobic mesophilic bacteria (plate count agar incubated for 72h at 30°C, DIN EN ISO 4833-2:2013), yeasts and moulds (Bengal red chloramphenicol agar, 7 days at 25°C, DIN EN ISO 21527-1:2008), Enterobacteriaceae (Crystal violet neutral red bile glucose agar, 24h at 37°C, DIN EN ISO 21528-2:2009-1), *Escherichia coli* (Tryptone Bile X-glucuronide agar, 4h at 37°C followed by 20h at 44°C, DIN EN ISO 16649-2:2001), *Clostridium perfringens* (Tryptose-sulfite-cycloserine agar, 20h at 37°C and anaerobiosis, DIN EN ISO 7937) and coagulase positive staphylococci (Baird-Parker agar, 48h at 37°C, DIN EN ISO 6888-1:1999) were evaluated through the traditional plate count method. Prevalence of *Salmonella* spp. (DIN EN ISO 6579), *Listeria monocytogenes* (DIN EN ISO 11290-1&2) and *Campylobacter* spp. (DIN EN ISO 10272-1) was also considered. MALDI-ToF MS (Matrix assisted laser desorption/ionisation time of flight mass spectroscopy) equipped with the AnagnosTes SARAMIS database (bioMérieux Deutschland GmbH, Nürtingen, Germany) was used for confirming the microbial identity²². All the analyses were carried out in duplicate and the results were mediated.

Statistical analyses

One-way ANOVA followed by Tukey HSD post hoc test was applied for computing statistical differences between treatments in terms of chemical quality, larvae performances and microbial composition. General linear model (GLM) with “quasipoisson” distribution was applied when absence of normality on model residuals was detected. GLM with binomial distribution was instead used for detecting differences on larval survival, while general linear mixed model with substrate replicates as random effect and observations nested within the same box was implemented for over-time larval growth evaluation. For all the analyses, significance level of 0.05 was used. Spearman's rank correlation test was applied for evaluating the effect of substrate chemical composition on larvae performance as well as of substrate fatty acid and amino-acid profiles on larval fatty

acid and amino-acid profiles. All the statistical analyses were implemented under R statistical environment version 4.1.0²³.

Results and Discussion

The aim of the present study was to evaluate the ability of BSFL to convert anaerobically digested ASW, as well as to investigate quality and safety of the produced insects.

BSFL growth and waste bioconversion

Growth curves of BSFL reared on the different substrates are displayed in figure 1. While no statistically relevant differences were computed on growth models between samples 0ASW, 25ASW, 50ASW and 75ASW ($P=0.174$), no growth was detected in substrate 100ASW over 12 days of composting. GLMM analyses carried out on the growth curves clearly showed that, excluding the 100ASW treatment, all the other substrates performed similarly during the first 6 days of rearing, showing a worsening of the larval growth in the substrates 75ASW in the following days ($P=0.012$). The main reason for these differences in growth, which resulted in significantly lower final larval weight in substrate 75ASW ($P=0.001$, table 2), can be attributed to the chemical composition of the ASW used in the present experiment. High amounts of ash and lignin and low levels of lipids and carbohydrates were indeed measured on this material, while protein level as well as hemicellulose and cellulose contents were not different from chicken feed (supplementary material S1-A). This composition might be due to the origin of the ASW as well as to the anaerobic digestion occurring during the stabilisation period in anaerobic settling ponds. ASW used in the present experiment were indeed originated by two carnivorous fish species, which traditionally are fed with diets rich in protein and poor in carbohydrates and lipids²⁴. It is therefore conceivable that manure from these species contained relatively low amounts of lipids and carbohydrates. The following anaerobic digestion might have further worsened such condition with anaerobic microorganisms responsible for consuming significant amounts of organic matter²⁵⁻²⁷, generating ash-rich material.

As BSFL growth is negatively correlated with the ash level in the diet^{28,29}, and proper equilibrium of proteins, lipids and carbohydrates is important for supporting larval development³⁰, it is conceivable that substrates rich in ASW resulted in poor larval growth.

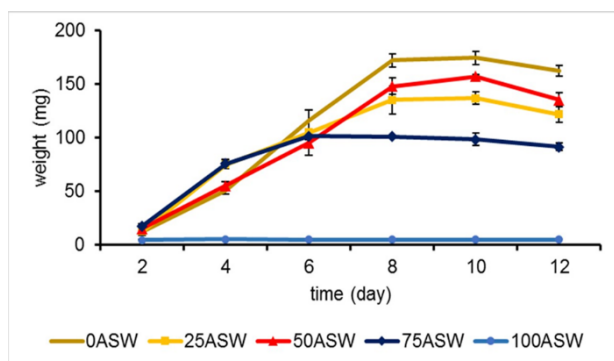


Figure 1. Averaged weight increase trend of 10 BSFL larvae reared on the experimental substrates. Error bars correspond to the standard error computed on 3 replicates/treatment.

Addition of increasing percentages of chicken feed to ASW led to a progressive reduction of ash ($P<0.000$), while the levels of carbohydrates ($P=0.003$) and lipids ($P<0.000$) increased (figure 2). Such conditions directly affected the larval growth (table 2) as suggested by the significant linear correlation (adjusted $R^2 = 0.9301$) computed between weight of ASW in diet and final larval weight (supplementary material S2).

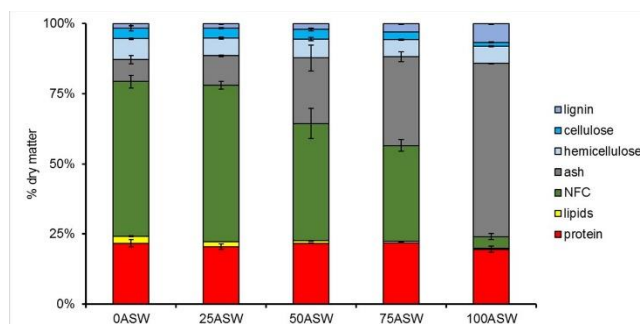


Figure 2. Chemical composition (% DM) of diets used for BSF larvae rearing. Error bars correspond to the standard error computed on 3 replicates/treatment.

Increasing lipids and carbohydrates levels in diet also resulted in a better substrate consumption by the BSFL, as suggested by the higher assimilated feed ($P=0.012$) computed for substrates 0ASW and 25ASW. However, despite an evident direct relationship was observed between ASW in diet and assimilated feed (table 2), no correlation was computed between this parameter and lipids or carbohydrates in diet (supplementary material S3-A). This suggests that other variables such as pH (Bohm et al., 2022) and Carbon to Nitrogen (C/N) ratio³¹ should also be considered. While pH was not evaluated in the present study, C/N ratio was stable between the different treatments, with values ranged between 10.32 and 13.20 (except for substrate 100ASW, supplementary material S1-A), which indicates high nutrient balance and therefore optimum conditions for BSFL growth and substrate utilization.^{32,33}

No direct correlation was computed between substrate reduction and chemical properties of the substrates, albeit a progressive worsening of performances was observed when higher levels of ASW were included in the diet. Noteworthy, the highest level of ASW inclusion (treatment 75ASW) resulted in a substrate reduction not significantly different from the substrate 0ASW, indicating that while high ASW in diet is not suitable for insect biomass production, it might be a viable possibility for ASW management. It might be explained by the higher larval survival and microbial activity recorded in this substrate. Several microorganisms are indeed known for degrading indigestible components of the diet, allowing a more exhaustive utilisation of the rearing substrate by the larvae¹⁰, while higher larval survival may indicate higher larval activity³⁴. However, while yeast and moulds recorded on substrates 75ASW were significantly lower than in the other substrates (table 6), suggesting low microbial contribution on substrate reduction, larval survival on the same treatment was significantly higher (table 2). Such condition, together with the low BCR and high FCR computed in substrates 75ASW, led to conclude that ASW was mainly indigested by the BSFL, which however were the main contributors to the substrate utilisation.

Table 2. Growth and bioconversion performances (average $\pm$ standard error, $n=3$) of BSFL reared on the experimental diets. Values within the same column followed by different letters are significantly different ($P<0.05$).

Treatment	Larval survival (%)	Final weight (mg)	Assimilated feed (g)	FCR	BCR (%)	Substrate reduction (%)
0ASW	83.17 $\pm$ 1.92 b	162.65 $\pm$ 4.81 a	42.35 $\pm$ 2.17 a	5.74 $\pm$ 0.75 b	6.89 $\pm$ 0.39 a	42.97 $\pm$ 1.26 a
25ASW	78.50 $\pm$ 1.00 b	121.82 $\pm$ 7.59 a	48.92 $\pm$ 13.91 a	8.82 $\pm$ 1.94 ab	5.07 $\pm$ 0.33 bc	43.95 $\pm$ 7.39 a
50ASW	82.67 $\pm$ 1.96 b	135.38 $\pm$ 6.53 a	31.23 $\pm$ 1.32 b	5.66 $\pm$ 0.25 b	5.78 $\pm$ 0.01 ab	35.27 $\pm$ 1.17 b
75ASW	91.83 $\pm$ 1.01 a	91.48 $\pm$ 3.38 b	37.46 $\pm$ 0.55 ab	13.11 $\pm$ 0.38 a	4.34 $\pm$ 0.20 c	42.08 $\pm$ 0.51 a

Nutritional quality of BSFL

Rearing substrate greatly affected the insects' proximate composition (table 3). Progressive decreases in dry matter ($P<0.000$), protein ($P<0.000$) and lipid ($P<0.000$) contents were noted when higher amounts of ASW were included in the diet. On the other hand, ash content increased ($P<0.000$), following a similar trend as the diet. Such observations are consistent with already published data, which shows high dependence of the insect chemical composition on the substrate chemical composition.³⁵⁻³⁷

While larval dry matter appeared for being strongly affected by several substrate parameters (supplementary material S3-A), with lower dry matter measured in insects growth on substrate with lower dry matter and energy content, larval fat was significantly correlated with volatile solids ($P=0.001$), lipids ($P<0.000$) and gross energy ($P=0.009$) contained in the substrate. Direct relationship was also detected between insect lipids and amount of carbohydrates in the diet ($P=0.024$), although correlation coefficient was lower than 0.7 (supplementary material S3-B). No correlation was observed between insects' lipids and dietary protein ($P=0.308$). These observations are in line with previous studies^{27,38-40} and confirm that BSFL are able to assimilate dietary lipids⁴¹ as well as to convert carbohydrates in lipids⁴². However, as the lowest lipid content was measured for the BSFL showing the lowest BCR, specific conditions such as adequate feed availability and dietary digestibility, should be

addressed. As a low BCR indicates ineffective consumption of the substrate⁴³, apparent food limitation might be hypothesized in treatment 75ASW. Such condition, which might be due to the high ash and indigestible fibres in the diet, has already been described as responsible for the production of larvae with low fat reserves⁴⁴ and seems to be satisfactory for explain the lower lipids in BSFL growth on substrate containing higher amounts of ASW.

Crude protein in BSFL produced in the present study were in line with previous researches, where values ranged between 35 and 49% were often recorded^{45,46}. Differences in protein content might be explained by the different larval growth efficiency observed in different substrates. Lower crude protein level was indeed computed for BSFL reared on substrate containing high ASW, i.e. the substrates that resulted in smaller and lighter larvae, and several studies have shown that protein level in BSFL increased fast in the first few days of life, and less during the fattening stage^{13,47}.

Ash level in larvae was observed to mimic the ash level in diet ($P<0.000$). It confirms the observation of Ewald et al. (2020)⁴⁸ and Liland et al. (2017)⁴⁹ for BSFL reared on mussel waste and algae. Similar results have also been obtained by Sprangers et al. (2017)²⁷ for BSFL reared on anaerobically digested vegetable waste and Rossi et al. (2023)¹³ when growing percentages of fresh ASW were included in the diet. The most suitable explanation for such differences should be found on the presence of some undigested substrate in the larval gut.³⁶

Table 3. Proximate composition (% dry matter, average $\pm$ standard error, $n=3$) of BSFL reared on different experimental diets. Values within the same row followed by different letters are significantly different ($P<0.05$).

Parameter	0ASW	25ASW	50ASW	75ASW
Dry matter (% fresh matter)	36.43 $\pm$ 1.02 a	33.28 $\pm$ 0.29 b	31.84 $\pm$ 0.15 b	22.24 $\pm$ 0.32 c
Lipids	35.52 $\pm$ 1.21 a	27.26 $\pm$ 1.63 b	26.07 $\pm$ 0.88 b	17.27 $\pm$ 0.52 c
Protein	40.62 $\pm$ 0.61 a	39.86 $\pm$ 0.55 a	37.54 $\pm$ 0.38 b	35.87 $\pm$ 0.34 b
Ash	11.03 $\pm$ 0.34 d	17.19 $\pm$ 0.61 c	22.58 $\pm$ 0.41 b	31.74 $\pm$ 0.08 a
NDF	12.84 $\pm$ 0.87	15.69 $\pm$ 1.40	13.81 $\pm$ 0.78	15.11 $\pm$ 0.42
ADF	8.54 $\pm$ 0.25	9.58 $\pm$ 0.52	8.14 $\pm$ 0.31	8.16 $\pm$ 0.18
ADL	1.60 $\pm$ 0.13	2.05 $\pm$ 0.14	1.74 $\pm$ 0.14	1.59 $\pm$ 0.14

Concerning the fatty acids profile, addition of ASW to the diet led to a reduction in SFA ($P < 0.000$) and increase in MUFA ($P < 0.000$) and PUFA ($P < 0.000$) (table 4). Despite of it, no critical effects were observed in insects, with only significant difference detected in BSFL raised on substrate 75ASW (table 4). Similar results were also obtained by Liland et al. (2023)¹¹ for BSFL reared on substrates containing increasing amounts of oven dried ASW, although raising amounts of ASW in diet led to an increase in MUFA but not PUFA in insects. On the other hand, in our study, BSFL produced on substrate richer in ASW did show approximately 20% more PUFA than BSFL from treatment 0ASW. This is in line with previous findings and confirm that fatty acid profile of BSFL can be modified by the diet⁵⁰, although correlation was only found for few fatty acids (supplementary material S3-C). However, although ASW appeared to be an important source of PUFA n-3 (supplementary material S1-B), and higher percentage of these fatty acids were measured in substrates richer of ASW (table 4), BSFL raised on substrates containing ASW did not show any increase of these fatty acids. Significantly higher concentration of PUFA n-6 were instead detected for larvae harvested in substrates with higher amounts of ASW ($P < 0.000$). These data, on one hand confirm the inability of BSFL to synthesize PUFA n-3⁴⁹, while on the other hand shown a strong preference of BSFL to accumulate PUFA n-6.

Considering individual fatty acids, lauric acid was the most abundant in all the larvae, even though it was quite low in substrates. This observation is in line with Fitriana et al. (2022)⁴⁶, confirming that lauric acid can be actively synthesized by BSFL. However, amount of lauric acid measured in BSFL from treatment 75ASW was approximately half of the others treatments (table 4). Such differences might be explained by the lower level of carbohydrates measured in substrate 75ASW (figure 2). Carbohydrates are the precursor of acetyl-CoA, a molecule crucial for the synthesis of lauric acid.⁴³ Despite this, since the amount of lauric acid measured in 75ASW insects was very high in comparison with the rearing substrate, simple bioaccumulation may not be a satisfactory

explanation. Alternative hypothesis might therefore be connected to a possible oxidation of UFA presents in the initial substrates.^{49,51} Lower amount of lauric acid in BSFL from the treatment 75ASW was offset by higher concentrations of palmitic and oleic acids, which were almost double that from insects harvested from the other substrates (table 4). Inverse correlation between lauric and palmitic acid in BSFL has already been observed by El-Dakar et al. (2021)⁵² and may be due to a metabolic adaptation connected to the low percentage of carbohydrates in the diet.⁵¹ The same explanation may also be adopted for oleic acid as significantly higher concentrations of this fatty acid ($P < 0.000$) have been found in BSFL raised on substrates containing high percentages of PUFA n-3 and low amount of carbohydrates.^{36,50} Metabolization and oxidation of PUFA n-3 might on the other hand be the main reason for the higher concentration of linoleic acid in BSFL from substrate 75ASW ($P < 0.000$). Several studies have indeed observed that PUFA n-3 are only accumulated during the first hours of life of BSFL, while a progressive degradation of these fatty acids was noticed when longer rearing times were applied.^{47,48,53}

Although statistical analyses showed some variability on amino acid profiles of BSFL (table 4), differences between samples were much lower than between substrates. No correlation was indeed found between levels of amino acids in larvae and substrates (supplementary material S3-D), suggesting that rearing substrate did not affect the amino acid profile of the larvae. Such observation is in accordance with Oonincx and Finke (2021)⁵⁴, who stated that amino acids in edible insects are independent of the diet. On the other hand, recent studies have shown the possibility to manipulate some amino acids as methionine, arginine, leucine, threonine, lysine, valine and tryptophan.^{37,55} The use of a uniform started feed during the first 6 days of life might be the explanation for the absence of difference on amino-acid profile in larvae from different substrates. BSFL are indeed known for synthesizing amino-acids at the beginning of their life, keeping the composition stable during the later stages.⁵⁶

Table 4. Fatty acid profile (% total fatty acids, average $\pm$ standard error, $n=3$) and amino acid profile (% crude protein, average $\pm$ standard error, $n=3$) of experimental substrates and BSFL reared on the correspondent experimental diet. Values within the same row followed by different letters are significantly different ($P < 0.05$).

Fatty acid ¹		0ASW	25ASW	50ASW	75ASW
Butyric (4:0)	substrate	<0.005	0.22 $\pm$ 0.01 b	1.12 $\pm$ 0.11 b	8.11 $\pm$ 0.76 a
	BSFL	<0.005	<0.005	0.02 $\pm$ 0.02 a	0.45 $\pm$ 0.40 a
Capric (10:0)	substrate	<0.005	<0.005	<0.005	<0.005
	BSFL	1.03 $\pm$ 0.01 a	0.98 $\pm$ 0.01 ab	0.92 $\pm$ 0.01 b	0.71 $\pm$ 0.02 c
Lauric (12:0)	substrate	0.25 $\pm$ 0.00 b	0.24 $\pm$ 0.01 b	0.27 $\pm$ 0.01 b	0.36 $\pm$ 0.01 a
	BSFL	56.13 $\pm$ 0.86 a	57.43 $\pm$ 0.62 a	56.93 $\pm$ 0.43 a	29.00 $\pm$ 0.84 b
Myristic (14:0)	substrate	0.24 $\pm$ 0.01 c	0.30 $\pm$ 0.00 bc	0.43 $\pm$ 0.01 b	0.82 $\pm$ 0.06 a
	BSFL	7.28 $\pm$ 0.11 b	8.27 $\pm$ 0.11 a	8.53 $\pm$ 0.06 a	5.83 $\pm$ 0.14 c
Myristoleic (14:1)	substrate	<0.005	<0.005	<0.005	<0.005
	BSFL	0.09 $\pm$ 0.00 c	0.08 $\pm$ 0.00 d	0.10 $\pm$ 0.00 b	0.15 $\pm$ 0.00 a
Pentadecanoic (15:0)	substrate	<0.005	<0.005	<0.005	<0.005
	BSFL	0.08 $\pm$ 0.00 c	0.10 $\pm$ 0.00 c	0.13 $\pm$ 0.00 b	0.45 $\pm$ 0.01 a
Palmitic (16:0)	substrate	16.88 $\pm$ 0.11 d	17.76 $\pm$ 0.17 c	19.11 $\pm$ 0.05 b	19.99 $\pm$ 0.17 a
	BSFL	9.46 $\pm$ 0.14 b	9.66 $\pm$ 0.11 b	10.28 $\pm$ 0.12 b	16.86 $\pm$ 0.29 a
Palmitoleic (16:1)	substrate	0.19 $\pm$ 0.00 b	0.25 $\pm$ 0.01 b	0.39 $\pm$ 0.01 b	0.79 $\pm$ 0.10 a

	BSFL	1.52 ± 0.03 c	1.58 ± 0.01 c	2.06 ± 0.03 b	6.63 ± 0.08 a
Margaric (17:0)	substrate	<0.005	<0.005	<0.005	0.14 ± 0.07 a
	BSFL	0.12 ± 0.00 c	0.14 ± 0.00 bc	0.16 ± 0.00 b	0.36 ± 0.01 a
Stearic (18:0)	substrate	2.73 ± 0.03 c	2.96 ± 0.11 bc	3.19 ± 0.07 b	4.09 ± 0.10 a
	BSFL	1.40 ± 0.02 b	1.39 ± 0.02 b	1.50 ± 0.01 b	3.52 ± 0.04 a
Elaidic (18:1 n-9 trans)	substrate	0.17 ± 0.02 b	0.19 ± 0.01 bc	0.24 ± 0.01 b	0.45 ± 0.03 a
	BSFL	0.06 ± 0.01 b	0.06 ± 0.01 b	0.12 ± 0.01 b	0.18 ± 0.02 a
Oleic (18:1 n-9 cis)	substrate	25.58 ± 0.30 a	25.59 ± 0.15 a	24.69 ± 0.02 a	21.81 ± 0.28 b
	BSFL	8.79 ± 0.25 b	7.78 ± 0.19 c	7.86 ± 0.12 bc	17.10 ± 0.29 a
Linoleic (18:2 n-6 cis)	substrate	49.85 ± 0.22 a	48.17 ± 0.12 b	45.39 ± 0.27 c	37.03 ± 1.10 d
	BSFL	12.88 ± 0.55 b	11.51 ± 0.41 bc	10.47 ± 0.18 c	16.65 ± 0.79 a
Arachidic (20:0)	substrate	0.31 ± 0.00 c	0.35 ± 0.00 bc	0.42 ± 0.01 b	0.63 ± 0.04 a
	BSFL	0.08 ± 0.00 c	0.09 ± 0.00 bc	0.09 ± 0.00 b	0.41 ± 0.01 a
Gondoic (20:1)	substrate	0.27 ± 0.01 c	0.29 ± 0.00 c	0.33 ± 0.00 b	0.44 ± 0.02 a
	BSFL	0.03 ± 0.00 a	0.02 ± 0.00 a	0.02 ± 0.00 a	<0.005 b
α-linolenic (18:3 n-3)	substrate	2.87 ± 0.05 a	2.89 ± 0.02 a	2.83 ± 0.01 a	2.38 ± 0.12 b
	BSFL	0.92 ± 0.03 a	0.76 ± 0.02 b	0.58 ± 0.01 c	0.69 ± 0.04 bc
Heicosanoic (21:0)	substrate	<0.005	<0.005	<0.005	<0.005
	BSFL	0.03 ± 0.00 b	0.07 ± 0.01 b	0.10 ± 0.00 b	0.42 ± 0.05 a
Eicosadienoic (20:2)	substrate	0.14 ± 0.01 c	0.15 ± 0.00 c	0.18 ± 0.00 b	0.24 ± 0.00 a
	BSFL	0.03 ± 0.00 a	0.02 ± 0.00 b	<0.005	<0.005
Behenic (22:0)	substrate	0.32 ± 0.02 c	0.38 ± 0.00 c	0.56 ± 0.02 b	1.08 ± 0.02 a
	BSFL	0.07 ± 0.00 b	0.06 ± 0.00 b	0.07 ± 0.00 b	0.38 ± 0.01 a
Di-omo-γ-linolenic (20:3 n-6)	substrate	<0.005 c	<0.005 c	0.22 ± 0.01 b	0.29 ± 0.02 a
	BSFL	<0.005	<0.005	<0.005	<0.005
Lignoceric (24:0)	substrate	0.20 ± 0.02 b	0.20 ± 0.00 b	0.25 ± 0.01 ab	0.50 ± 0.08 a
	BSFL	<0.005	<0.005	0.02 ± 0.00 b	0.11 ± 0.00 a
EPA (20:5 n-3)	substrate	<0.005	<0.005	<0.005	0.07 ± 0.07 a
	BSFL	<0.005	<0.005	0.02 ± 0.00 b	0.10 ± 0.01 a
Nervonic (24:1)	substrate	<0.005	0.08 ± 0.04 b	0.17 ± 0.01 b	0.29 ± 0.03 a
	BSFL	<0.005	<0.005	<0.005	<0.005
DHA (22:6 n-3)	substrate	<0.005	<0.005	0.21 ± 0.02 b	0.58 ± 0.02 a
	BSFL	<0.005	<0.005	<0.005	<0.005
SFA	substrate	20.93 ± 0.11 d	22.41 ± 0.20 c	25.36 ± 0.22 b	35.73 ± 1.23 a
	BSFL	75.69 ± 0.82 a	78.19 ± 0.60 a	78.76 ± 0.34 a	58.51 ± 1.10 b
UFA	substrate	79.07 ± 0.11 a	77.59 ± 0.20 b	74.64 ± 0.22 c	64.27 ± 1.23 d
	BSFL	24.31 ± 0.82 b	21.81 ± 0.60 b	21.24 ± 0.34 b	41.49 ± 1.10 a
MUFA	substrate	26.20 ± 0.28 a	26.39 ± 0.20 a	25.82 ± 0.04 a	23.77 ± 0.12 b
	BSFL	10.49 ± 0.26 b	9.52 ± 0.19 b	10.17 ± 0.15 b	24.05 ± 0.34 a
PUFA	substrate	52.87 ± 0.18 a	51.21 ± 0.14 b	48.82 ± 0.27 c	40.50 ± 1.11 d
	BSFL	13.83 ± 0.58 b	12.29 ± 0.42 bc	11.07 ± 0.19 c	17.44 ± 0.84 a
PUFA n-6	substrate	50.00 ± 0.23 a	48.32 ± 0.12 ab	45.79 ± 0.26 b	37.47 ± 1.08 c
	BSFL	12.91 ± 0.55 b	11.53 ± 0.41 bc	10.47 ± 0.18 c	16.65 ± 0.79 a
PUFA n-3	substrate	2.87 ± 0.05 b	2.89 ± 0.02 ab	3.04 ± 0.03 a	3.03 ± 0.03 ab
	BSFL	0.92 ± 0.03 a	0.76 ± 0.02 b	0.60 ± 0.01 c	0.78 ± 0.06 ab
Amino acid ²					
Aspartic acid	substrate	9.06 ± 0.19 b	9.41 ± 0.24 b	9.75 ± 0.09 ab	10.16 ± 0.08 a
	BSFL	9.99 ± 0.20	9.39 ± 0.29	9.50 ± 0.14	10.20 ± 0.08
Threonine	substrate	3.50 ± 0.04 c	3.53 ± 0.07 bc	5.35 ± 1.47 b	4.10 ± 0.03 a
	BSFL	4.87 ± 0.11 a	3.67 ± 0.15 b	3.98 ± 0.03 b	3.91 ± 0.08 b

Serine	substrate	4.84 ± 0.09 b	4.91 ± 0.10 b	5.17 ± 0.08 ab	5.36 ± 0.04 a
	BSFL	4.29 ± 0.09 a	3.30 ± 0.13 b	3.54 ± 0.15 b	4.34 ± 0.13 a
Glutamic acid	substrate	21.70 ± 0.43 a	20.18 ± 0.06 b	18.62 ± 0.18 c	15.59 ± 0.22 d
	BSFL	10.85 ± 0.56 b	9.84 ± 0.68 b	10.65 ± 0.43 b	14.32 ± 0.24 a
Glycine	substrate	4.44 ± 0.06 c	4.76 ± 0.06 c	5.35 ± 0.12 b	6.14 ± 0.13 a
	BSFL	6.37 ± 0.16 ab	6.07 ± 0.10 b	6.10 ± 0.04 b	6.90 ± 0.22 a
Alanine	substrate	4.95 ± 0.06 c	5.29 ± 0.04 c	5.89 ± 0.05 b	6.80 ± 0.21 a
	BSFL	7.51 ± 0.22 b	7.41 ± 0.32 b	7.43 ± 0.06 b	9.64 ± 0.62 a
Valine	substrate	4.79 ± 0.03 c	5.02 ± 0.01 b	5.40 ± 0.06 a	5.63 ± 0.07 a
	BSFL	7.27 ± 0.22	7.22 ± 0.14	7.10 ± 0.07	7.57 ± 0.11
Cysteine	substrate	1.72 ± 0.02	1.66 ± 0.14	1.60 ± 0.02	1.60 ± 0.04
	BSFL	0.64 ± 0.05 b	0.63 ± 0.00 b	0.74 ± 0.02 b	1.18 ± 0.09 a
Methionine	substrate	1.39 ± 0.02	1.33 ± 0.10	1.24 ± 0.07	1.13 ± 0.07
	BSFL	1.90 ± 0.12	1.84 ± 0.04	1.93 ± 0.05	1.75 ± 0.07
Iso-leucine	substrate	4.12 ± 0.08 b	4.23 ± 0.06 ab	4.48 ± 0.05 ab	4.35 ± 0.04 a
	BSFL	4.99 ± 0.13 b	5.02 ± 0.05 ab	5.05 ± 0.04 ab	5.40 ± 0.08 a
Leucine	substrate	7.97 ± 0.10	8.10 ± 0.07	8.23 ± 0.08	8.03 ± 0.06
	BSFL	7.71 ± 0.20	7.42 ± 0.16	7.39 ± 0.07	8.02 ± 0.12
Tyrosine	substrate	3.23 ± 0.05	3.12 ± 0.02	3.28 ± 0.05	3.37 ± 0.07
	BSFL	5.72 ± 0.05 a	4.29 ± 0.28 b	4.94 ± 0.33 ab	5.55 ± 0.37 ab
Phenylalanine	substrate	4.97 ± 0.04	5.13 ± 0.04	5.17 ± 0.04	5.05 ± 0.07
	BSFL	4.79 ± 0.11	4.51 ± 0.06	4.54 ± 0.03	4.70 ± 0.07
Histidine	substrate	2.58 ± 0.03	2.56 ± 0.03	2.51 ± 0.08	2.36 ± 0.03
	BSFL	3.57 ± 0.08	3.07 ± 0.14	3.09 ± 0.12	3.10 ± 0.22
Lysine	substrate	4.73 ± 0.13	4.66 ± 0.14	4.81 ± 0.08	4.89 ± 0.04
	BSFL	6.19 ± 0.07 b	6.14 ± 0.21 b	6.43 ± 0.12 ab	7.11 ± 0.29 a
Arginine	substrate	5.85 ± 0.12 a	5.68 ± 0.17 ab	5.54 ± 0.02 ab	5.28 ± 0.03 a
	BSFL	4.94 ± 0.19 a	4.02 ± 0.11 b	4.53 ± 0.17 ab	4.93 ± 0.14 a
Proline	substrate	6.88 ± 0.18 a	6.90 ± 0.11 a	6.49 ± 0.09 a	5.67 ± 0.05 b
	BSFL	6.24 ± 0.21	6.18 ± 0.21	6.04 ± 0.09	6.41 ± 0.13

¹ Capronic (6:0), Caprylic (8:0), Undecanoic (11:0), Pentadecenoic (15:1), Margaroleic (17:1), Linoleilaid (18:2 n-6 trans), γ -linolenic (18:3 n-6), Erucic (22:1 n-9), Eicosatrienoic (20:3 n-3), Tricosanoic (23:0), Arachidonic (20:4 n-6), Docosadienoic (22:2) acids were measured but not detected neither in substrates nor in BSFL (limit of detection = 0.005%)

² Taurine and Hydroxyproline were measured but not detected neither in substrates nor in BSFL (limit of detection = 0.05%)

Microbial evaluation of BSFL

Microbial analyses performed in the present study showed that anaerobically digested ASW is an important source of microorganisms, especially Enterobacteriaceae and anaerobic bacteria (supplementary material S1-D). Occurrence of *L. monocytogenes* was observed in pure ASW, while no *Salmonella* spp. was found in ASW. Mixing chicken feed to ASW led to a progressive dilution of these microorganisms, although total viable counts, as well as coagulase positive Staphylococci was not severely affected (table 5). Interestingly, while *L. monocytogenes* was detected on one of the three samples from substrate 75ASW, *Salmonella* spp. was only found and confirmed in all the samples from substrate 0ASW (supplementary material S4).

According to the microbial contamination of the rearing substrate, high total viable counts were also detected in all the obtained insects

with not apparent effect of the rearing substrate (table 5). Such high contamination, which is in line with previous studies^{57,58}, can be explain by the absence of an inactivation treatment and by the presence of the gut.^{59,60}

No significant difference was observed in Enterobacteriaceae and coagulase positive Staphylococci counts, while increasing counts of *E. coli* were measured for BSFL raised on substrates with higher ASW levels (table 6). Such microorganisms have already been reported in edible insects⁶¹ and may raise safety concerns if not properly considered. However, while occurrence of Staphylococcus spp. was not confirmed in any samples, *E. coli* was clearly identified (supplementary material S4). Presence of this species, which has been often found on edible insects, might be explained by the absence of a post-harvest sanitisation treatment.⁵⁹

Presumptive *C. perfringens* were observed in all the samples (table 5), with contamination levels extremely variable between the treatments ($P < 0.000$). However, MALDI-ToF analyses did not allow any confirmation (supplementary material S4), leading us to conclude that *C. perfringens* was not present in the studied material. No *Salmonella* spp. were detected in any larvae sample, although these species were found in the OASW substrate. Such result is in accordance with Wynants et al. (2019)⁵⁷ and Osimani et al. (2021)⁵⁸, who also did not observe presence of *Salmonella* spp. in fresh larvae reared on organic waste. On the other hand, De Smet et al. (2021)⁶²

and Grisendi et al. (2022)⁶³ recorded high *Salmonella* spp. counts in BSFL reared on chicken feed artificially contaminated by *Salmonella*. Since microbial contamination of BSFL as well as their antimicrobial activity is extremely variable and highly affected by rearing substrate and environmental conditions^{57,64}, we cannot conclude that *Salmonella* spp. will never be detected on BSFL reared on ASW. Microbial inactivation treatments should therefore be always carried out before allowing any use of BSFL, regardless by their rearing substrate.

Table 5. Microbial counts (log CFU/g, average $\pm$ standard error, $n=3$) of experimental diets. Values within the same column followed by different letters are significantly different ($P < 0.05$).

Treatment		Total viable count	Yeasts & Moulds	Enterobacteriaceae	<i>Escherichia coli</i>	<i>Clostridium perfringens</i>	Coagulase positive Staphylococci
OASW	Substrate	8.18 $\pm$ 0.02 c	5.49 $\pm$ 0.03 a	7.96 $\pm$ 0.06 ab	3.18 $\pm$ 0.02 a	1.30 $\pm$ 0.47 c	5.30 $\pm$ 0.10
	BSFL	8.39 $\pm$ 0.05	4.47 $\pm$ 0.13	8.14 $\pm$ 0.20	3.73 $\pm$ 1.25 b	5.61 $\pm$ 0.88 a	7.76 $\pm$ 0.11
25ASW	Substrate	8.74 $\pm$ 0.04 b	5.23 $\pm$ 0.19 a	8.16 $\pm$ 0.05 a	3.39 $\pm$ 0.14 a	5.45 $\pm$ 0.01 b	5.24 $\pm$ 0.14
	BSFL	8.86 $\pm$ 0.31	5.57 $\pm$ 0.83	8.24 $\pm$ 0.14	5.23 $\pm$ 0.43 a	2.94 $\pm$ 0.53 b	8.00 $\pm$ 0.18
50ASW	Substrate	8.84 $\pm$ 0.04 ab	3.47 $\pm$ 0.31 b	7.87 $\pm$ 0.12 ab	1.00 $\pm$ 0.33 b	6.07 $\pm$ 0.07 a	5.68 $\pm$ 0.13
	BSFL	8.99 $\pm$ 0.38	2.85 $\pm$ 0.95	8.40 $\pm$ 0.23	5.62 $\pm$ 0.68 a	4.92 $\pm$ 0.16 a	7.73 $\pm$ 0.38
75ASW	Substrate	8.97 $\pm$ 0.01 a	3.49 $\pm$ 0.09 b	7.75 $\pm$ 0.08 b	<1	6.52 $\pm$ 0.03 a	5.65 $\pm$ 0.07
	BSFL	9.28 $\pm$ 0.12	>5	7.72 $\pm$ 0.16	>5	>5	7.31 $\pm$ 0.17

L. monocytogenes was detected on BSFL harvested from substrate 50ASW. However, although MALDI-ToF analyses confirmed the occurrence of *Listeria* spp., it did not offer any confirmation at species level (supplementary material S4), leaving room for discussion. Indeed, while several non-pathogenic *Listeria* species have already been detected on edible insects, *L. monocytogenes* has never been detected.⁶⁵ To the best of the authors' knowledge, the only studies where occurrence of *L. monocytogenes* was reported consisted in challenge experiments using artificially contaminated substrates.⁶³ Since the ASW used in the present study were naturally contaminated with *L. monocytogenes*, it cannot be assumed that this species is completely absent in the harvested insects, although previous studies suggests low chances of occurrence.

Conclusions

Although current legislation does not allow to rear insects on waste, the present study shows that anaerobically digested ASW can be managed by BSFL. However, compared with previous studies where fresh ASW was used, lower conversion ability was observed, suggesting that treatment of fresh ASW should be preferred. The main reason for the lack of insects growth observed in pure ASW (substrate 100ASW) was attributed to the anaerobic digestion, which resulted in low lipid and carbohydrate levels and high ash content, while the protein level remained constant. Addition of an amending material acting as main source of carbohydrates and lipids has been shown to improve BSFL growth and enhance bioconversion performances. Mixing ASW and CF in 1:1 ratio (substrate 50ASW) did not show any significant reduction in larval growth, although lower

feed assimilation and substrate reduction was recorded. BSFL reared on substrate containing increasing concentrations of ASW displayed acceptable nutritional quality with regard to protein and lipid contents, although levels of crude protein and fat were lower than in insects reared on pure chicken feed. Amino-acid and fatty acid profiles were not severally affected, albeit significant differences were computed for insects raised on substrate 75ASW. High microbial counts were detected in all the produced insects, regardless of the rearing substrate. No pathogens were detected; however, their presence could not be neglected. A sanitisation treatment prior to further use was therefore advisable.

Author contributions

Giacomo Rossi: Conceptualization, Data curation, Investigation, Methodology, Investigation, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing.

Shikha Ojha: Methodology, Supervision, Writing – review & editing.

Julia Hankel: Data curation, Methodology, Validation, Writing – review & editing.

Oliver K. Schlüter: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Conflicts of interest

"There are no conflicts to declare".

Data availability

The data supporting the findings of this study are available within the article.

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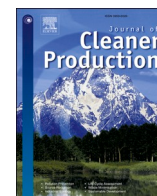
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Estimating the dynamics of greenhouse gas emission during black soldier fly larvae growth under controlled environmental conditions

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Novel food

ABSTRACT

Edible insects are considered a sustainable alternative protein source. Several studies have indicated that greenhouse gas (GHG) emissions from insects are generally low. These data were usually recorded in closed static chambers, which did not reflect the actual rearing conditions. On the other hand, open respiration chambers need high amounts of substrate and insects, making the analyses time consuming and expensive. In the present study, a novel small-scale open gas exchange recording system for evaluation of GHG emissions from insects is presented. The device consists of four independent respiration chambers, each one equipped with a pump for supplying air, a flow meter and a septum for sampling the flowing air. A validation experiment was carried out using black soldier fly larvae as model organisms and moistened chicken feed (dry matter 40%) as rearing substrate. Three independent runs were conducted, each with two replicates, using 200 neonates larvae and 300 g of substrate. Moistened chicken feed (300 g) without larvae was used as control. Throughout the 12 days rearing period, gas sampling occurred every 12 h. Data interpolation considering a linear tendency between two successive sampling points was applied and overall amounts of gases were quantified as area under the curve. Irrespective of the run, CO₂ emissions in the insect-substrate systems increased during the initial 7 d of experiment, consistently followed by a rapid decrease. Multiple cross correlation analysis showed high reproducibility ($r \geq 0.9$), while significant differences in the CO₂ emissions between insects-substrate system (range 7.76–11.88 g g⁻¹ dry insects) and sole substrate (range 0.20–0.33 g g⁻¹ initial dry substrate) were observed. Very limited N₂O (≤ 1.13 mg g⁻¹ dry insects) and CH₄ (≤ 1.33 mg g⁻¹ dry insects) emissions were detected in this experiment, indicating that the performed black soldier fly larvae rearing did not result in significant production of these GHGs. Overall, the experiment showed that the novel device may be particularly suited for quick and reliable estimation of GHG emissions from edible insects.

1. Introduction

Approximately 30% (15.8 Gt CO₂-eq) of the overall (52.5 Gt CO₂-eq) global greenhouse gas (GHG) emissions are generated by the agro-food system (Li et al., 2022). Excluding the non-food agriculture, the food supply chain alone is responsible for 13.7 Gt emissions (Poore and Nemecek, 2018), with 7.4 Gt of CO₂-eq originating within the farm gate (crops and livestock production) (FAO, 2022). Emissions from meat production are estimated to be approx. 2.8 Gt of CO₂-eq per year, accounting for 39% of the overall agriculture GHG emissions (excluding transport and land use) (Li et al., 2022). A large part of such emissions is shared among ruminant animals with cattle and sheep responsible for 30

and 24 kg of CO₂-eq per kg of meat, respectively (FAO, 2022). Hence, it is evident that the modern food system is unsustainable and requires transformation.

Novel and future food sources have been described as perfect means for ensuring the ecological transition of the food system while safeguarding the nutritional quality of the diet (Parodi et al., 2018). Whether integrated with other novel food sources, inclusion of edible insects in the daily diet may represent the turning point toward a sustainable food system (Mazac et al., 2022).

Research conducted to date has demonstrated that insects can efficiently grow on waste, by-products and decaying materials while emitting very low GHG (Moruzzo et al., 2021). Despite insect rearing is

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typically associated with low gas emissions (Boakye-Yiadom et al., 2022), only a few studies investigating the GHG emissions of edible insects have been published so far. Moreover, with only few exceptions (Parodi et al., 2020), most of these studies investigated the gas emissions in closed static chambers (Ermolaev et al., 2019; Mertenat et al., 2019). Although such systems can be useful for shaping a first idea on the insects' gas emissions, they cannot be considered entirely accurate since they do not simulate the actual insect growing conditions. Accumulation of CO₂ and moisture, and depletion of O₂ within such chambers may indeed lead to metabolic modifications, which, concomitantly, affect gas emissions (Akbar et al., 2016; Boakye-Yiadom et al., 2022; Mertenat et al., 2019). On the other hand, open systems (Matyssek and Herppich, 2020) may be difficult and costly to operate, needing huge amounts of substrate and insects to detect very low concentrations of relevant gases (Parodi et al., 2020). Therefore, in order to measure insects' gas emissions efficiently and cost-effectively, to compare various substrates and to offer important information to entrepreneurs and policy makers, small scale and efficient gas emissions monitoring systems need to be developed. The aim of this study was to design and test a new emission chamber for efficiently recording the GHG emissions of insects, while simulating real rearing conditions. This paper presents and describes the new device in the first part. In the second section, results of a validation experiment are discussed. Black soldier fly larvae (BSFL) and moistened chicken feed were used as model organisms.

2. Description of the gas emission chamber

The innovative climate-controlled gas exchange system (Fig. 1a) for continuous recording of insects' gas emissions was based on a small-scale respiration chamber for measuring the metabolic activity of fresh vegetables (Linke et al., 2010; Rux et al., 2017). The new system consisted of four acrylic glass cylinders (gas exchange chamber, V = 9.42 L, H = 30 cm, Ø = 20 cm, Fig. 1b) in which boxes with substrate and insects could be housed. Each cylinder could be hermetically sealed by a removable front acrylic glass push-fit cap (Ø = 22 cm, W = 1 cm) fitted with polyurethane frame closure connection (2.5 cm height). The closing system consisted of a rectangular aluminium rod (H = 3 cm, L = 24.5 cm, W = 1 cm) and a polyurethane-screened aluminium cylinder (Ø = 5.5 cm, W = 1 cm). In each chamber, three sensors recorded temperature, relative humidity and air pressure ((d), ALMEMO FHAD 46-C2 multi-sensor module, Ahlborn Mess-und Regelungstechnik GmbH, Holzkirchen, Germany). Sensors, placed along the vertical central line of each unit and fixed through screw IP68 polyamide Cable Glands connectors (Conrad Electronic SE, Hirschau, Germany), were connected to a PC. Data were recorded at 1 min interval and analysed

with the help of an Almemo-COM-based program (written by C. Regen, ATB, Potsdam, Germany).

Continuous airflow through each chamber was obtained by an inlet-outlet system. The inlet system (Fig. 1, green pipes (a)), consisting of PVC tubes (inside diameter (iØ) = 1.5 cm), led temperature and humidity conditioned air from a climate chamber into a PVC sampling box (H = 19 cm, L = 10.5 cm, W = 12 cm) equipped with a polyisoprene 11 mm septum (Sigma-Aldrich, St. Louis, Missouri, USA) for gas sampling and further to a second central input PVC box (H = 19 cm, L = 10.5 cm, W = 12 cm). Through smaller silicon tubes (iØ = 1.0 cm), four pumps ((b), Daypower Luftpumpe LP36-12, Pollin Electronic GmbH, Pförring, Germany) pushed the inlet air into each gas exchange chamber (c). Flow rate of each inlet line was independently controlled and regulated by adjusting the voltage of the pump.

For the outlet system (Fig. 1a, red pipes (e)) 1.0 cm-silicon tubes (overall length 210 cm) were placed on the front cap of each chamber and fixed through a 4 cm long polyamide semi-flexible pipe (Landefeld Druckluft und Hydraulik GmbH, Kassel, Germany). At 200 cm, a Rotilabo T connector (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), provided with a natural polyisoprene 11 mm septum ((g), Sigma-Aldrich, St. Louis, USA) was installed for gas sampling. An analogue flowmeter ((f), placed on the final section of the outlet tube was finally responsible for recording airflow rates.

In order to ensure exact temperature control, the entire system was placed inside a climate chamber (Bio Line VB1014, Vötsch Industrietechnik GmbH, Balingen, Germany) from which the inlet air was conditioned. Besides PC and power supply, flowmeters, inlet sampling box and outlet tubes including the outlet sampling septa were placed outside the climate chamber.

3. Materials and methods

3.1. Substrate and insects

Commercial chicken feed (all-mash A Mehl M.Cocc., Deuka companion GmbH, Düsseldorf, Germany) with an initial dry matter content of $91.62 \pm 0.07\%$ was ground (10000 rpm) into a fine powder with a knife mill (Retsch GmbH, Hann, Germany), applying three cycles of 10 s each, separated by breaks of 30 s to homogenise the bulk. Aliquots (133.5 g) of this powder were transferred into a transparent 2 L polypropylene box (Ikea Deutschland GmbH & Co. KG, Munich, Germany) and moistened at approx. 60% of humidity by adding 166.5 g of distilled water (final substrate mass 300 g). To avoid excess of free water on the surface, prepared substrates were kept two days (48 ± 2 h) at 10 °C before adding larvae. For each run, four boxes with substrate were

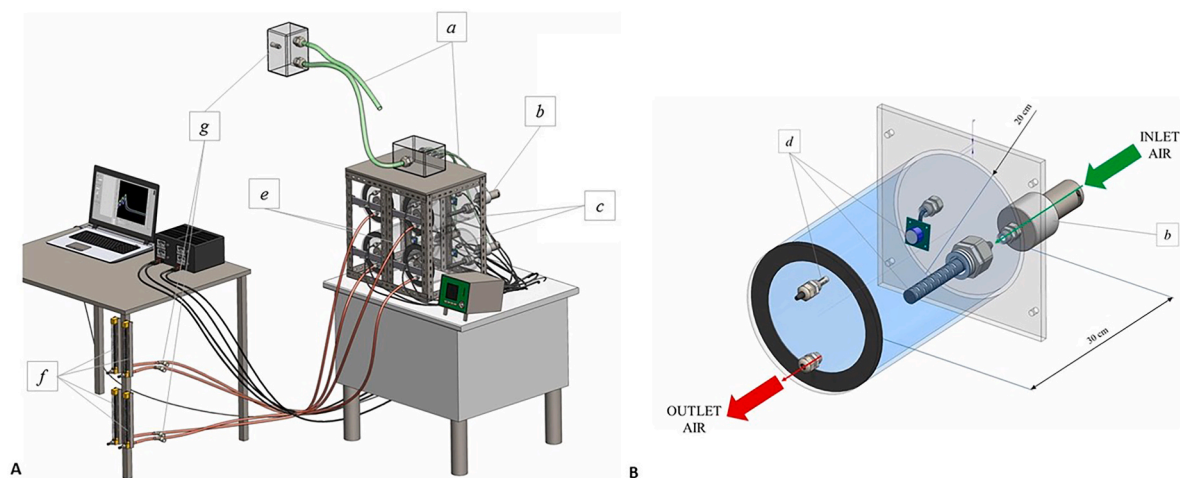


Fig. 1. Overview of the gas exchange system for online recording of insects' GHG emissions (A) and overview of the specification of the gas exchange chamber (B). SolidWorks 3D CAD 2021 professional design software was employed for drawing the device.

prepared but insects were added only in two boxes (treatments: T1, T2), while the other two boxes were used as control (C1, C2).

Black soldier fly larvae (BSFL, *Hermetia illucens*) were chosen as model insects. Neonates BSFL (less than 12 h old), generated from eggs (Hermetia Baruth GmbH, Baruth/Mark, Germany) laid on corrugated cardboard were used. Eggs were left to hatch without substrates in a polypropylene transparent box placed in a climate chamber ($30 \pm 1^\circ\text{C}$, $70 \pm 5\%$ RH, 0:24 L:D). Eggs eclosion started approximately 36 h after receiving the eggs and only the larvae originated in the following 12 h (36–48 h from the purchase) were used in the experiment. Exactly 200 neonate larvae were carefully transferred in two of the four boxes with substrate through a camel brush. All the boxes were closed with a polypropylene transparent lid (Ikea Deutschland GmbH & Co. KG, Munich, Germany) equipped with a rectangular hole ($15\text{ cm} \times 10.5\text{ cm}$) screened with polyester black mosquito net ($1\text{ mm} \times 1\text{ mm}$ mesh, HaGa-Welt GmbH & Co. KG, Nordstemmen, Germany). Number of BSFL and amount of feed were carefully defined with the aim of ensuring approximately 125 mg of fresh feed/larvae/day or 50 mg of dry feed/larvae/day as suggested by [Diener et al. \(2009\)](#) and [Dzepe et al. \(2021\)](#). After adding larvae, all boxes were immediately transferred into individual gas exchange chambers, placed inside a Bio Line climate chamber ($27 \pm 1^\circ\text{C}$ and $70 \pm 5\%$ RH). Each gas exchange chamber was hermetically closed, drain pumps switched on and data recording initialized. No further feed was provided during the experiment.

3.2. Experimental set-up and gas emission measurements

Experiments were repeated three times (three runs). Two independent replicates were performed for each run (six replicates in total).

Actual concentration of N_2O , CH_4 and CO_2 emitted by insects and substrates were discontinuously measured through GC analysis of the air sampled from the inlet and outlet systems. Gas sampling was carried out every 12 h (every day at 8:00 a.m. and 8:00 p.m. ($\pm 30\text{ min}$)), along the entire insect rearing period of 12 d. At each sampling point, three gas samples were sampled with a 25 mL gastight glass syringe (1025 TLL, PTFE Luer Lock, Hamilton Company, Nevada, USA) equipped with a Sterican Gr2-G21 $0.8\text{ mm} \times 40\text{ mm}$ needle and immediately conveyed in a pre-evacuate (2.7 mbar of negative pressure) 25 mL glass vial equipped with a PTFE/butyl rubber septum stopper.

Sampling started approximately 10 h after setting up the box inside each gas exchange unit (stabilization period = $10 \pm 1\text{ h}$). CO_2 and O_2 concentrations, relative humidity and pressure inside each unit were monitored before and after each sampling and the airflow rates were adjusted to keep the relative humidity inside the unit between 65 and 75% RH.

To minimise the interaction with external environment, gas exchange units and climate chamber were not opened during the experiments. After 12 d, all gas exchange chambers were opened and the rearing boxes were collected. BSFL were harvested from T1 and T2 treatments by handpicking them with forceps, carefully cleaned with towel paper, counted and weighted on an analytical balance (CPA224S-OCE, Sartorius AG, Göttingen, Germany). Mass of residuals (frass from T1 and T2, substrate residuals from C1 and C2) was also recorded.

3.3. Gas quantification

Air samples were analysed every Monday, Wednesday and Friday morning. When needed, air samples were dark-stored in a BD-S 115 safety cabinet (Binder GmbH, Tuttlingen, Germany) at 20°C to minimise any possible external impact. Air samples were analysed with a GC-2014 gas chromatograph (Shimadzu Deutschland GmbH, Duisburg, Germany) equipped with FID and ECD detectors as well as a HS-20 auto sampler. Four different Agilent stainless steel packed columns (Haysep N, 1.0 m; Haysep D, 4.0 m; Mole Sieve 5A, 2.5 m; Haysep N, 1.5 m) were used (overall length 9 m, inner diameters 2 mm and mesh size 80/100). Nitrogen was used as carrier gas, while hydrogen and synthetic gas

Alphagaz 1 (Air Liquide Deutschland GmbH, Berlin, Germany) were the fuel gasses for FID.

Air samples (2 mL) from the vials were directly injected into the GC. Gas flow was set at 25 mL min^{-1} , oven temperature at 80°C , and FID and ECD temperatures at 250°C and 325°C , respectively. CH_4 concentration (detection range: 1–100 ppm) was measured directly from the sample, while CO_2 was converted to CH_4 within a methaniser operating at 380°C , prior detection by FID (detection range: 1–100000 ppm). N_2O concentration was finally measured by ECD (detection range: 0.05–100 ppm). Effective gas concentration (ppm) was calculated by the LabSolutions LCGC software (Shimadzu Deutschland GmbH, Duisburg, Germany) using four standard calibration gas mixtures (Air Liquide Deutschland GmbH, Düsseldorf, Germany). For each studied gas, intra-run accuracy of the GC was computed as average of the absolute difference between the recorded value and the average of three independent determinations performed on the same standard mixture.

3.4. Chemical analyses of substrates, larvae and residuals

Dry matter (DM), volatile solids (VS), ash, total nitrogen (N), total carbon (C), total organic carbon (TOC), total sulphur (S) contents, and gross energy were analysed on substrates, 12 d old-insects and residuals. Additionally, ammonium ($\text{NH}_4\text{-N}$), nitrate ($\text{NO}_3\text{-N}$) and nitrite ($\text{NO}_2\text{-N}$), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents were determined in substrates and residuals. Insects were analysed after lyophilisation, while substrates and residues after oven-drying at 60°C for 48 h. DM and ash contents were determined gravimetrically after oven-drying at 105°C overnight (NFTA 2.1.4 official moisture method) and incineration at 550°C for 4 h (DIN 38414-EN 12879), respectively. VS content was calculated by difference between DM and ash. Total N content was determined with the Kjeldahl method (AOAC, 2000-method 992.15), while C and S were analysed with the element analyser VARIO EL (Elementar Analysensysteme GmbH, Langenselbold, Germany), following the producer's instructions. TOC was gravimetrically determined by quantifying the amount of carbon released after sample combustion in acidic environment (DIN-EN 13137:2001–12), while total protein content was computed by multiplying the N content with 4.76 for insects ([Janssen et al., 2017](#)) and 5.70 for substrates ([Sriperum et al., 2011](#)). Gross energy was determined on the samples dried at 105°C for 24 h, following the oxygen bomb method (ISO 9831:1998), while NDF, ADF, ADL contents were analysed according to the ISO 13906:2008 and AOAC 973.18 methods, using the Ankom 2000 Fiber Analyser (Ankom Technology, Macedon, USA). Finally, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ contents were quantified with the ion chromatography system Dionex ICS-1000 (Thermo Fisher Scientific, Waltham, USA) after samples extraction with distilled water ($\text{NO}_2\text{-N}$) or 0.0125 M CaCl_2 solution ($\text{NH}_4\text{-N}$ and $\text{NO}_3\text{-N}$). Chemical determinations of substrates were performed in triplicate, while insects and residues were analysed in duplicate. In both cases, results from the same sample were averaged.

3.5. Calculations, data modelling and statistical analysis

Larvae growth and substrate reduction performances of insects were computed through the following indices:

- (1) Bioconversion rate (BCR; %) = (total final larvae mass (g)/total substrate mass (g)) * 100
- (2) Assimilated feed (g DM) = total substrate dry mass (g) – total frass dry mass (g)
- (3) Feed conversion rate (FCR) = (assimilated feed (g))/(final larvae dry mass (g))
- (4) Substrate reduction (%) = (assimilated feed (g)/substrate dry mass(g)) * 100
- (5) Larval survival (%) = (final larval number/initial larval number) * 100

As the experiments started with 12 h old-larvae, initial larval mass was assumed to be zero.

Differences in gas concentrations (outlet – inlet) were multiplied by flow rates (L min^{-1}) and modelled through interpolation between subsequent data points. The overall volume (mL) of each gas emitted from each single chamber was estimated by calculating the area under the curve. Computed volume of each gas was then converted into mass (g) by multiplying it with the ratio of molar mass of the respective gas (CO_2 : $44.009 \text{ g mol}^{-1}$; N_2O : $44.013 \text{ g mol}^{-1}$; CH_4 : $16.043 \text{ g mol}^{-1}$) and the molar volume of an ideal gas (22.41 L mol^{-1}). Intra run and inter run reproducibility were therefore computed through multiple cross correlation analysis.

Two-samples *t*-tests ($P < 0.05$) with observations nested within the same run was applied for comparing the overall amount of each gas emitted by the system substrate + insects with the amount of gas emitted by the sole substrate. Additionally, in order to evaluate if gas concentration in outlet and inlet air were effectively different, differences in gas concentration were compared with the accuracy of the GC. Differences were therefore considered reliable only if they exceeded the accuracy of the measuring device.

One-sample *t*-test was finally used to evaluate whether emissions of N_2O and CH_4 were significantly different from zero.

Data modelling, interpolation and area under the curve procedure were performed in MATLAB R2021b (The MathWorks Inc., Natick, MA, USA), while statistical analyses were conducted on R software version 4.2.3 (R-CoreTeam, 2023; Vienna, Austria).

Chemical data of substrates, insects, frass (consisting in residual substrate, larvae excreta and larvae exuviae from treatments T) and residues (substrate residual from treatments C) were used for computing the absolute mass (g) of each component, which were applied for

calculate the overall mass balances as described by Parodi et al. (2020). To this end, the mass of each element emitted as gas (g) was calculated by multiplying the overall mass of the gas (g) by the ratio of molar mass of the considered element ($12.011 \text{ g mol}^{-1}$ for C and $14.007 \text{ g mol}^{-1}$ for N) and the molar mass of the respective gas. Element distribution between larvae, residual and air as well as percentage of not recovered element (element lost) were computed through the following equations (Parodi et al., 2021):

- (6) Element in larvae (%) = (element in larvae (g)/element in substrate (g)) * 100
- (7) Element in residual (%) = (element in residual (g)/element in substrate (g)) * 100
- (8) Element in gas (%) = (element in emitted gas (g)/element in substrate (g)) * 100
- (9) Element lost (%) = $100 - (\text{element in larvae (\%)} + \text{element in residual (\%)} + \text{element in gas (\%)})$

4. Results and discussion

The aim of this investigation was to test and validate the new gas exchange chamber. Three independent runs and two replicates per run evaluating the GHGs emitted by BSFL reared on moistened chicken feed were carried out. Therefore, emissions of the system substrate + insects (treatment T) were compared with the emission of the sole substrate (treatment C), and experimental reproducibility was evaluated through a multiple cross correlation analysis.

Although GHG emissions from insects are known to be highly variable (Parodi et al., 2020), the recovered data showed high inter-run reproducibility, suggesting suitability of the novel device for rapid and

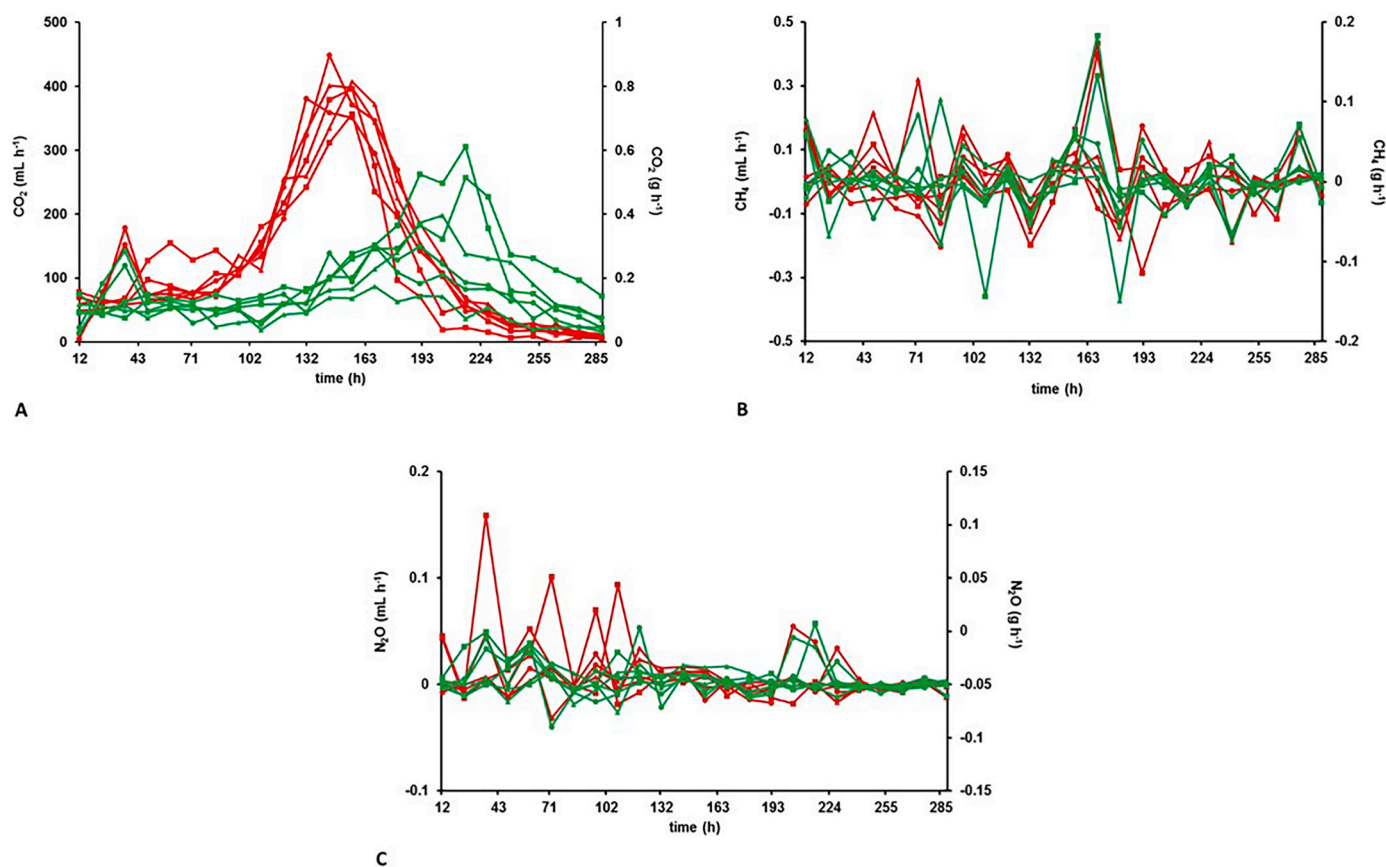


Fig. 2. Gas emission curves for CO_2 (A), CH_4 (B), N_2O (C) recorded during the experimental runs. Red curves represent the gas emitted by the substrate + insects systems (treatments T) while green curves indicated the gas emitted by the sole substrate (treatments C). Different markers are used for different run: RUN-1 = square, RUN-2 = round, RUN-3 = triangle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reliable estimation of insect gas emissions.

4.1. GHG emissions trends and experimental reproducibility

CO₂ emissions from the treatments T (Fig. 2A, red curves) were low at the beginning, increased after approximately 3.5 d, peaked between day 5 and day 7 and decreased again up to days 8–9. Such bell-shaped trend was observed in all the runs, with very good overlapping of curves. Cross correlation analysis (supplementary material S1) displayed a strong significant positive intra and inter run correlation ($r \geq 0.9$) between treatments T, which suggested high reproducibility of the process. The aforementioned dynamics have already been noticed by other authors (Bekker et al., 2021; Ermolaev et al., 2019; Pang et al., 2020a; Perednia et al., 2017), further supporting the validity of the measuring procedure. The main explanation for this particular dynamic might be found in the activity of BSFL and in the nature of the rearing substrate. CO₂ is mainly originated from microbial and larval metabolism (Boakye-Yiadom et al., 2022) whereby it is plausible to expect low emissions at the beginning, followed by a fast increase and a final reduction and stabilization. Bekker et al. (2021) also observed that with a substrate moisture of 65–75%, larvae started producing significant CO₂ only after reaching 5–6 mg of mass. After this point, linear correlation between CO₂ production and larval mass was usually observed (Laganaro et al., 2021). In the present experiment, larvae less than 12 h old were used. Consequently, they could have had a very low activity during the first hours of experiment, explaining the low CO₂ emission observed at the beginning. As the larvae grew, their metabolic activity (i. e., respirational activity) increased (Gligorescu et al., 2019), resulting in higher CO₂ release. Similarly, the reduction of CO₂ release observed at the end of each run could suggest a decline in larvae metabolic activity, mainly due to the achievement of the critical mass (i. e., the minimum mass to allow metamorphosis) as well as to a substantial reduction of the readily available feed (Bekker et al., 2021; Gligorescu et al., 2019). Chemical analyses of substrate before and after (frass) the BSFL growth are shown in supplementary material S2. Considering the different dry matter recorded for substrate and frass, a reduction of approximately 50% of total volatile solids was detected. It was accompanied by a significant decline in TOC (47–53%) and NDF (15–24%) contents, while absolute amounts of ADF and ADL increased. Different from NDF, which comprises cellulose, hemicellulose and lignin (Hindrichsen et al., 2006), ADF and ADL represents the amount of only hemicellulose and lignin (Peguero et al., 2023). While cellulose is known to be easily degraded by BSFL, hemicellulose and lignin are not directly digested (Gold et al., 2018), pointing out that after 12 d of BSFL composting, no easily digestible substrate was available. On the other hand, although the insects might have had already reached a satisfactory weight for going through the metamorphosis, they still appeared far from the pupal stage, suggesting that provision of new fresh feed (re-feeding) could have reactivated the metabolism, resulting in higher final larval weight and a new CO₂ emission peak (Zhang et al., 2021).

CO₂ emissions from the sole substrates (Fig. 2A, green curves, treatments C) were roughly stable during the first week of the experiment and increased slightly only after this period. In these substrates, CO₂ emissions could mainly be associated to microbial activity (Oonincx et al., 2010) as high fungal growth was observed in every run. Microbial activity is well known for playing a crucial role in substrate modification and CO₂ emissions (Ermolaev et al., 2019). Future studies should focus on understanding the effect of microorganisms on the GHG emissions of substrate with and without BSFL.

Concerning CH₄ (Fig. 2B) and N₂O (Fig. 2C), no clear dynamics were observed for either treatments T or C. The absence of consistency on such data was also confirmed by the cross correlation, which highlighted no correlation at the inter-run level, although some positive correlations were observed within the same run (supplementary material S1). One sample-t-test (supplementary material S3) and comparisons between effective gas concentration and accuracy (supplementary material S4) of

the measuring device showed that no difference from zero were statistically observed, suggesting that BSFL did not emit any CH₄ or N₂O in our experiment. It is consistent with previous reports, which highlighted very low or absence of production of such GHGs by BSFL (Boakye-Yiadom et al., 2022; Perednia et al., 2017), which is strictly dependent on the physico-chemical characteristics and nature of the rearing substrate (Chen et al., 2019; Ermolaev et al., 2019; Sandrock et al., 2019; Pang et al., 2020a; Mertenat et al., 2019).

4.2. Cumulative gas emissions and mass balance

Cumulative gas emissions highlighted that BSFL were mainly responsible for producing CO₂, while no significant emissions of CH₄ and N₂O could be detected (Table 1). It is in accordance with previous studies that suggested very limited CH₄ and N₂O emissions, compared with direct CO₂ emissions (Lindberg et al., 2022).

Two-sample *t*-test carried out on the overall dataset detected significant difference between the system substrate + insects and the sole substrate only for CO₂ ($P = 0.001$), suggesting that BSFL were the main contributors for the CO₂ emissions of the system. An increase in CO₂ emissions due to BSFL activity was also observed in the BSFL-mediated composting process of manure when compared to classical composting (Parodi et al., 2021). On the other hand, no statistically significant differences between treatments T and C were computed for CH₄ ($P = 0.406$) and N₂O ($P = 0.428$) cumulative emissions, confirming that production of such gasses was mainly due to microbial activity on the substrate, as already stated by Oonincx et al. (2010) and Parodi et al. (2021). However, although significant differences in CO₂ emissions were detected across the entire experiment, the intra-run ANOVA showed significant differences between treatment T and treatment C only in RUN-2 ($P = 0.002$). This highlighted that other parameters, such as environmental conditions, feeding status and larval metabolic activity might have influenced the overall GHG emissions from insects. Possible explanation for the no statistical significance in runs 1 and 3 could indeed be the low number of larvae compared with the high amount of substrate used in the present study as well as the already mentioned high microbial activity. The later might also be the main explanation for the higher amount of N₂O recorded from the treatment T in RUN-1, compared to the other runs. Furthermore, it should be noted that, although not important differences were observed on the substrate composition between different runs (supplementary material S2), the insects reared on RUN-1 showed lower growth and slightly higher survival (Table 2). These two aspects together might have affected the substrate turning activity, resulting in different air circulation within the bulk and promoting the selection of specific microbial species, particularly active on denitrification and N₂O production, as already observed by Nigussie et al. (2016) in heartworm-mediated composting processes. Chemical analyses of frass (supplementary material S2) originated along the three runs support such statement. Although an increase in nitrate and nitrite from substrate to frass was observed in all the run, the amounts of both nitrate and nitrite measured in frass from the two treatments in RUN-1 were significantly lower than those measured in the frass from the other two runs. Since N₂O is primarily originates from nitrate and nitrite reduction (Thamdrup, 2012), lower concentration of these compounds in frass may suggest higher denitrification. On the other hand, it is also interesting to observe that the amount of nitrite in residuals of treatments C from RUN-1 was significantly higher than the amount of nitrite in residuals generated in runs 2 and 3. This suggests that the denitrification process and overall N₂O production are more influenced by general environmental conditions rather than by the presence and activity of BSFL. However, it should be mentioned that such phenomena may not be universally applicable as higher larval density and/or lower feeding rate could also impact the microenvironment inside the substrate (Chen et al., 2019).

CO₂ cumulative emissions computed as g of gas per kg of dry insects, ranged between 11880 and 7760 (Table 1). Despite the high variability

Table 1

Gas emissions (CO₂, N₂O, CH₄; mean ± standard error, n = 2) of the substrate + insects systems (treatments T) and sole substrate (treatments C). Values within the same run followed by different lowercase letters are statistically different (P < 0.05). Values within the same column followed by different uppercase letters are statistically different (P < 0.05).

		CO ₂		N ₂ O		CH ₄	
		experimental (g) ^a	g-gas/g-insect (DM) ^b	experimental (mg) ^a	mg-gas/g-insect (DM) ^b	experimental (mg) ^a	mg-gas/g-insect (DM) ^b
RUN-1	T	64.34 ± 3.12 ^A	9.36 ± 0.44	7.62 ± 1.14	1.13 ± 0.27	9.10 ± 0.12	1.33 ± 0.11
	C	58.79 ± 1.92 ^B	–	4.74 ± 1.47	–	7.92 ± 0.70	–
RUN-2	T	73.75 ± 1.38 ^{aA}	11.88 ± 3.79	4.41 ± 0.52	0.74 ± 0.32	4.99 ± 0.29	0.79 ± 0.23
	C	41.37 ± 0.49 ^{bB}	–	4.74 ± 0.73	–	5.91 ± 0.13	–
RUN-3	T	72.88 ± 0.27 ^A	7.76 ± 1.14	2.62 ± 0.21	0.28 ± 0.02	7.45 ± 2.13	0.76 ± 0.11
	C	35.58 ± 9.71 ^B	–	2.19 ± 0.50	–	4.80 ± 0.40	–

^a Experimental: overall gas emitted by the system from the beginning to the end of the experimental run, calculated as area under the emission curve.

^b Computed by dividing the experimental gas recovered (g) from the treatment T (substrate + insects system) by the final insect dry mass measured from the same treatment.

Table 2

Growth and bioconversion performances (mean ± standard error, n = 2) of BSFL reared on moistened chicken feed.

	BCR (%)	FCR	Assimilated feed (g)	Substrate reduction (%)	Larval survival (%)	final larval mass (mg) ^a
RUN-1	6.04 ± 0.84	8.02 ± 0.07	55.32 ± 4.78	46.87 ± 4.05	97.00 ± 0.00	93.44 ± 13.06
	6.62 ± 1.95	9.20 ± 2.44	58.26 ± 4.49	49.11 ± 3.78	85.50 ± 6.00	114.28 ± 26.17
RUN-2	8.22 ± 1.26	5.43 ± 0.33	51.69 ± 4.33	46.60 ± 3.90	95.07 ± 2.07	125.87 ± 13.51
	8.22 ± 1.26	5.43 ± 0.33	51.69 ± 4.33	46.60 ± 3.90	95.07 ± 2.07	125.87 ± 13.51

^a Fresh mass of one single larva.

observed between the three runs, which may be explained by the uncontrolled microbial contribution, as well as by different insects' performances, cumulative CO₂ production was always significantly higher than in other studies (Boakye-Yiadom et al., 2022). Several aspects including the frequency of gas measurements, the number of reared larvae, the rearing substrate and the abiotic conditions should be considered to explain such difference. For example, Parodi et al. (2020) observed that increasing the gas sampling frequency resulted in higher cumulative CO₂ detection, thereby decreasing the evaluation errors. Additionally, Pang et al. (2020b) and Chen et al. (2019) showed that CO₂ emissions were positively correlated with substrate C/N ratio and moisture, respectively, suggesting that the substrate plays an important role in determining the actual GHG emissions. Additionally, it should be mentioned that 12 h old larvae were used in the present study, while other studies usually used 3 d, 5 d, 7 d or 10 d old larvae (Boakye-Yiadom et al., 2022). As young larvae are more active in terms of metabolic performances (Gligorescu et al., 2019), it could be hypothesised that neonate larvae produced more CO₂ than older larvae. This justifies the higher emission detected in the present study and suggests that emissions from BSFL in other studies are probably underestimated. In the present study, CO₂ cumulative emissions computed per mass unit of dry insects did not seem to be strongly related to the larval growth performances, although good correlation was computed between larval gained mass and CO₂ emission (Spearman's rank correlation: $\rho = 0.89$, $P = 0.0333$). Low correlation was instead detected between substrate reduction and CO₂ emission (Spearman's rank correlation: $\rho = 0.26$, $P = 0.6583$), as well as between CO₂ emission and FCR (Spearman's rank correlation: $\rho = -0.60$, $P = 0.2417$). It confirms the findings of Chen et al. (2019), Eriksen (2022) and Laganaro et al. (2021), while contradicts Ooninx et al. (2010) and Lindberg et al. (2022), who observed an inverse correlation between FCR and CO₂ production and a direct

correlation between substrate reduction and CO₂ production.

CH₄ emissions varied between 760 and 1328 mg kg⁻¹ of dry BSFL (Table 1). Such values were within the range of published data (between 5.5 and 10066 mg kg⁻¹ of dry BSFL) (Boakye-Yiadom et al., 2022), and confirms that methane production relies more on the rearing substrate than on the BSFL. Indeed, high CH₄ emission is only observed when manure or decaying material is used (Parodi et al., 2021), while it is negligible when chicken feed is the rearing substrate (Perednia et al., 2017). However, it should be noted that our values are higher than already published values on commercial feed (Parodi et al., 2020). Such difference might be explained by the different experimental set-up applied in our study. Specifically, while Parodi et al. (2020) reared 30000 larvae on 12 kg of substrate, a very limited larvae/substrate ratio (200 larvae on 300 g of feed) was applied in our study. In such conditions, we might hypothesise that anoxic areas were generated inside the substrate, favouring the growth of methanogenic bacteria, which may have produced CH₄ (Chen et al., 2019). Similar considerations could also be valid for N₂O. Emissions of this GHG indeed usually range between 0.33 and 118 mg kg⁻¹ of dry larvae (Boakye-Yiadom et al., 2022). Significantly higher N₂O emissions were recorded in our experiment, ranging between 275 and 1130 mg kg⁻¹ of dry larvae (Table 1). The higher N₂O emissions measured in the present study might be explained by the high feeding rate (i.e. 0.6 g of dry substrate/larvae or 50 mg of dry feed/larvae/day) as higher feeding rate has been associated to higher N emissions (Nigussie et al., 2017; Rossi et al., 2023). The high variability observed in the present experiment was mainly due to the RUN-1. Indeed, as already mentioned, smaller larvae were collected in such treatments. The reduced mobility of smaller larvae within the substrate could lead to lower oxygen dissolution and higher denitrification (Thamdrup et al., 2012), which is also supported by the lower concentration of nitrate and nitrite in the frass (supplementary information 2).

Despite the differences in cumulative gas emitted across the three runs, the mass balance of C and N in substrate + insects treatments displayed an overall recovery of approximately 93% and 85–95%, respectively (Figs. 3a and 4a). Similar recovery was also observed for C in the sole substrate treatments (92–93%; Fig. 3b), while higher variability was detected for N (80–99%; Fig. 4b). Although such values are still far from 100%, based on the observations of Parodi et al. (2020) and Pang et al. (2020a) they can still be considered satisfactory. Specifically, Parodi et al. (2020) observed an overall recovery rate of 95% for C and 101% for N by using a continuous air detection system, while Pang et al. (2020a) were able to recover 87.48–97.90% of the initial C and 74.40–98.39% of the initial N, when BSFL were reared on food waste.

In the present study, the mentioned recovery rates were similarly to those of these studies (Pang et al., 2020a; Parodi et al., 2020) and the variability on gas emission quantification was lower than in Mertenat et al. (2019). This indicates that the novel gas emission chamber works and presents high capability for large-scale implementation. However, while noteworthy performances were reached for C, the result remained

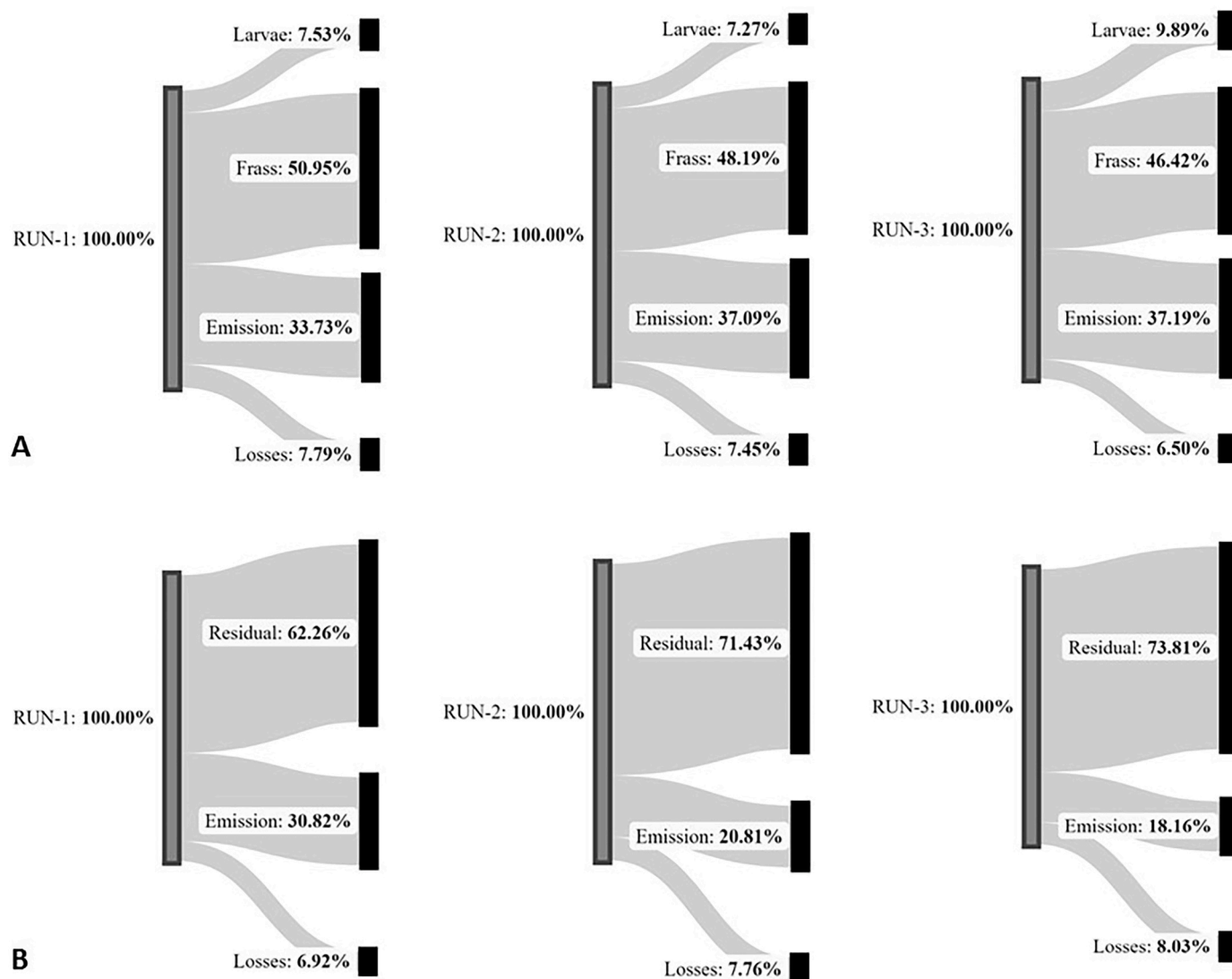


Fig. 3. Mass balance of Carbon along the three experimental runs from the substrate + insects system (A) and the sole substrate treatment (B).

low for N. The main reason for the low recovery of N might be associated to sampling frequency and accuracy of the detection method (Pang et al., 2020a), as well as the absence of a NH_3 detection system. Although NH_3 is not a direct GHG, it is often considered as main responsible for water and soil pollution (IPCC, 2014). Additionally, it might be oxidised to N_2O (FAO, 2006; Iwasa et al., 2015) contributing to increase its level in air. As NH_3 production is usually favoured by high temperature, elevated pH and increased substrate aeration (Pang et al., 2020a; Zhang et al., 2021), it can be hypothesised that significant production of such gas occurred, especially during the first days of conversion (Chen et al., 2019). It was further suggested by the strong acrid smell perceived at the end of the outlet system as well as by the significant increase of $\text{NH}_4\text{-N}$ concentration observed in both, T and C treatments (supplementary material S2). $\text{NH}_4\text{-N}$ may originate from protein degradation and mineralisation, as well as from nitrite reduction (Alvarez et al., 2014; Kroupina et al., 2022). While nitrite reduction is hardly conceivable in the studied conditions, mineralisation of protein can be hypothesised. Considering that $\text{NH}_4\text{-N}$ production is known to be responsible for increased NH_3 emissions (Yan et al., 2018; Liu et al., 2019), it is reasonable to conclude that significant NH_3 production occurred in the present experiment.

Different NH_3 production could be the reason for the high variation in the total N recovery of all treatments. Production of this gas is

extremely variable and depending on several factors, such as aeration rate, moisture, temperature and pH (Groot Koerkamp, 1994). Although detection of these factors was continuously carried out during the three experimental runs, no significant variations were observed (supplementary material S5) between the runs, excluding a potential effect of the environment on the overall NH_3 emissions. On the other hand, it has been hypothesised that different BSFL populations have different ability on producing NH_3 with not all the individuals being able to do it (Parodi et al., 2020). Another explanation could be that NH_3 emissions from BSFL rearing were strongly impacted by the larval metabolic performances (Parodi et al., 2021) as well as by the substrate microbial activity (Gold et al., 2018). Specifically, as BSFL grow, significant amounts of uric acid are produced, which are typically converted into NH_3 by microorganisms (Green and Popa, 2012).

High variability on percentage of N recovery was also observed in the treatment C (Fig. 4b) indicating different intensities of nitrification processes in the substrates. The analysed different contents of NO_2^- , NO_3^- and $\text{NH}_4\text{-N}$ in the substrates (supplementary material 2) indicate the volatilisation of a significant amount of $\text{NH}_4\text{-N}$ as NH_3 (Ren et al., 2018).

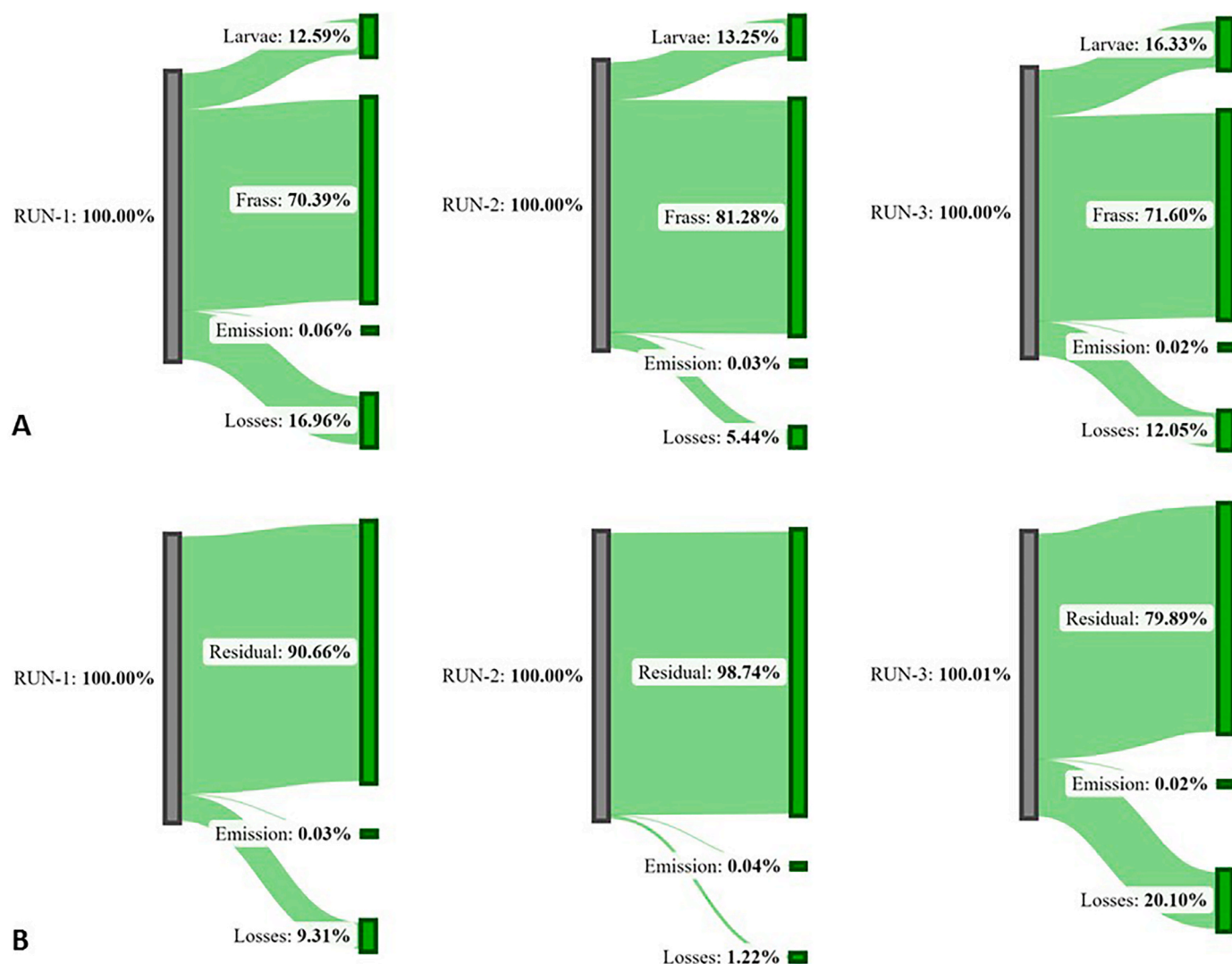


Fig. 4. Mass balance of nitrogen along the three experimental runs from the substrate + insects system (A) and the solo substrate treatment (B).

5. Conclusions

The present study showed that the novel gas emission chamber with continuous airflow is well suited for fast and reliable estimations of GHG emissions from edible insects. Although it was a first trial using the new device, gases emitted by BSFL grown on chicken feed were detected with high reproducibility in terms of CO₂ emission rate. However, the same level of reproducibility was not observed for CH₄ and N₂O emissions, which were generally limited and substrate dependent. Cumulative emissions computed as g g⁻¹ (gas/dry insect) were significantly higher than previously reported values, likely due to the use of younger larvae in this experiment. However, the absence of a continuous measurement method and the assumption that the airflow was linear between two successive measuring points might have led to overestimation of the emissions. Acceptable overall C recovery was detected for all runs, with values not significantly different from the ones reported in literature. Rather low N recovery percentage was instead detected as no NH₃ measurement was implemented.

Future studies should aim to improve the device by increasing the number of chambers in order to increase the number of parallel repetitions, implementing high-accuracy continuous emission detection systems and developing a NH₃ quantification method. Other substrates such as waste and manure, where CH₄ and N₂O emissions have already been detected, should also be tested with the aim of validate the device

for gases other than CO₂. Moreover, since the system showed low feed conversion efficiency and significant energy loss, further improvements on the experimental design, such as reduction of insects to substrate ratio, longer rearing period or repeated feeding events should be considered.

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CRediT authorship contribution statement

Giacomo Rossi: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Shikha Ojha:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Werner Berg:** Writing – review & editing, Visualization, Methodology, Investigation, Data curation. **Werner B. Herpich:** Writing – review & editing, Resources, Methodology,

Investigation, Formal analysis, Data curation, Conceptualization. **Oliver K. Schlüter**: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jclepro.2024.143226>.

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Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

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List of Scientific Publications

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