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Composition and Biological Activity of Edible Insects Biomasses: Effect of Rearing Conditions, Processing, and Gastrointestinal Digestion on Black Soldier Fly Raw Materials

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Abstract

By exploiting and adequately orienting what insects normally do in nature – i.e., growing on organic waste and using the available compounds for their metabolic needs and for their development – it is possible to design systems to recycle and reuse organic residuals. To date, one of the most studied species, due to its potential in bio converting organic by-products and waste, is the Black Soldier Fly (BSF, *Hermetia illucens* L.). With particular attention to this species, the present PhD thesis is aimed to better understand the potentiality of insects as food and feed ingredients, and to provide improved tools to fully exploit this potential. With the purpose of optimizing bioconversion performances of black soldier fly by minimizing wastes, we tested different seasonality mixes of agri-food by-products as growing substrates for insects' development, and we evaluated the effect on their nutritional composition with a focus on protein fraction. Black soldier fly species proved to be suitable in growing on basically all low-value agri-food wastes tested, simultaneously reducing waste, and producing valuable protein biomass that can be employed for numerous purposes. The most effective substrates that were identified were those belonging to the Autumn group. Furthermore, to fully achieve the sustainability objective, the composition of biomasses obtained from *lactobacillus* fermentation of black soldier fly wastes was determined. The nutritionally improved composition after fermentation proved that this process can be a way to valorize insect residual biomasses otherwise unused. This PhD thesis also presents a technical solution to the problem of chitin quantification in insect biomasses. An optimized UPLC-ESI/MS method for the simultaneous quantification of chitin and proteins was validated, providing a quicker and more accurate method to fully characterize insect biomass. The investigation on the effects on human health when insects are used as a high-quality and sustainable protein source was also another important issue that was addressed. As a result of in-vitro experiments, black soldier fly prepupa showed the ability at the intestinal level to inhibit the DPPIV enzyme and stimulate GLP-1 hormone release, suggesting an antidiabetic potential. In conclusion, the results obtained from this thesis improve knowledge on black soldier fly as a tool to re-use agri-food wastes and highlight the

high nutritional quality and potential beneficial effects on human health of insect-derived biomasses.

CHAPTER 1

General introduction

1.1 Sustainable food production in a growing world: the role of circular economy and waste management

1.1.1 Effects of the demographic changes and future perspectives

From 1950 to today, the world population has increased considerably. In the past 65 years, it passed from 2,5 billion to 7,5 billion, raising the world output of final goods and services by over 12 times (Dasgupta, A., & Dasgupta, 2021). Scientific research and technological development have been instrumental in this phenomenon. Progress in medicine and biology, public hygiene, and deep economic and social upheavals played a key role in extending life expectancy and enhancing its quality all around the globe. Considering the progress made until now in the technology and economic sector, and in biomedical research, with considerable therapeutic prospects, we expect further population growth in the next half-century (Dumont, 1994; Lutz & Qiang, 2002). Specifically, it is estimated that the planet will handle an additional 2,5 billion people by 2070 (Bongaarts, 2009). However, this second wave of demographic growth is supposed to be less intense than the previous one, and quite different. According to the projections of the United Nations (UN, 2000), it will be predominantly concentrated in urban areas and will face the problem of aging populations (Cohen, 2002). This further projected “demographic transition” inevitably will come with certain social, economic, and environmental consequences. If, on one hand, we can consider the increase in life expectancy and technological innovation extremely positive developments in the current situation, on the other, this perspective poses many ecological-related challenges for the future. The earth planet from 1950 onwards has already suffered several changes and environmental stresses related to the “population overshoot” phenomenon. It will be forced to face even more pressures from now on, principally because of further demographic growth and increase in per capita demand for goods and services. The relation between total population, consumption, and environment is transparent. Overpopulation leads to overconsumption and to more invasive human activities. This, of course, involves pollution of the environment and a depletion of basic resources (Pimentel et al., 1994). The quality and quantity of the natural resources (air, water, soil) is increasingly threatened and affected by a growing trend in the rate of

urbanization, expansion of transport network, large scale industrial and energy development, as well as higher agricultural outputs. Moreover, there is a significant lack of appropriate environmental norms and disposal wastes procedures. All these pressures are having and will have a severe impact on climate change and, consequently, on life forms' health and survival (Arora, 2018; Sivarethinamohan et al., 2020). United States Development Authority (USDA) and Organization for Economic Cooperation and Development (OECD) estimate that, by 2050, the planet will face a 0.2 ºC rise in the average temperature; a continued human action aimed at consuming resources will almost definitely result in loss of biodiversity and catastrophic consumption of natural resources; innovative responses to manage over 25 billion tons of solid wastes predicted by 2050 will need to be found (Arora, 2018). Thus, all the advantages of today's innovation will cause large, long-term losses. In view of all this, governments should implement practical and sustainable steps to respond adequately to this future reality, in order to ensure sufficient natural resources available for future generation needs. A realistic strategy could be created by changing the way we use natural resources (Yumurtacı, 2014).

1.1.2 A different way of managing the planet's resources

In the last century until recently, a linear economic model has been mostly followed. The abundant material and low-cost resources resulting from industrialization were intensively exploited to produce goods without taking into consideration a recycling plan for the several wastes generated. Eurostat (2011) reported that, in 2010, of the almost 65 billion tons of materials commercially exploited, over 3,5 percent (meaning 2,7 billion tons) were destroyed, even if potentially reusable. An approach more environment-conscious is needed to avoid all the negative effects on the environment and living beings' health deriving from bad waste management. The circular economy model was suggested by Pearce and Turner (1989), even if the potentialities of a cyclical ecological scheme with respect to the linear pattern were already discussed in previous years (Pearce & Turner, 1989; Sariatli, 2017). This system suggested a change in the concept of waste. A single use with subsequent direct disposal of resources was no longer expected to occur. Based on the Circular economy, materials should be saved with a view to subsequent reuse and

recycling, helping strategically to balance the consumption of energy and raw resources. The adoption of an economic model based on the principles of sustainable resource use is the only way for future generations to still support a development process compatible with the natural resources that the planet can offer (Sauvé et al., 2016).

1.1.3 Recycling as solution to the scarceness of agri-food resources

Combining the idea of sustainability and development by reducing the need to depend excessively on raw resources, which do not support the estimated population gain, became immediately a real challenge. During the United Nations General Assembly of 2015, the Member states presented a list of sustainable development goals to be reached by 2030 (UN, 2015), including reduction of poverty, improvement of the standard of health and education, and promoting economic growth without sacrificing our ecosystem. In this context, point 12 of the "Agenda for Sustainable Development" should receive special attention. In one of its points (12.3), it is stated: "By 2030, halve per capita global food waste at the retail and consumer levels and reduce food losses along production and supply chains, including post- harvest loss" (UN, 2015). The agro-food sector is one of the most threatened. Indeed, to respond adequately to the increase in the world's growing population wealth and demand (often exceeding the real needs) of food and natural resources, the agriculture and animal production system suffered very strong pressures. Hence, during the years, a significant reduction of the soil for agriculture and livestock farming was registered. From recent evaluations, it was estimated that to date more than 30% is no longer available and, with the growing trend in population and demands, a further 55% is expected to be gradually degraded, leading to an inevitable increase in cost production, which not all countries will be able to support (FAO & ITPS, 2015). Therefore, the need to adopt sustainable methods of food production and consumption is not only finalized to a reduction of food waste excess, which leads to environment pollution (Cuéllar & Webber, 2010; FAO, 2013). A change in the approach is also essential to avoid biodiversity loss risk, the reduction of the surface area for cultivating and rearing animals and dietary lacks in the poorest areas of the world with respect to the industrialized regions, owing to the inability to sustain the increased food production costs (Berry et al.,

2015). As the statistical database of the Food & Agriculture Organization of the United Nation has highlighted, a difference in the nutritional balance is already present among the poorest populations (Asia and Africa) and those industrially developed (i.e., America). It has emerged that, for instance, the per capita per year meat consumption in the poorest countries is approximately a quarter of the amount consumed by the richest populations (FAO, 2020). A growing trend in population numbers and decreasing number of natural resources will heighten this disparity if a linear economic model continues to be used. For all these reasons, the rural economic system should urgently change in an environmentally friendly way, starting from preventing food waste surplus. It was estimated that, just in the United States, food surplus derived waste results, in economic terms, in a loss of around 161 million USD per year (Mourad, 2016). In this context, there are two scenarios of action: limiting food over-output (with the consequent reduction in generating waste) or adopting environmentally appropriate solutions to manage leftovers generated by the intensive current food production system. However, the current high consumers' requests and the attention to food safety issues hardly allow to act only on the excess production of food, which is the source of the problem (Papargyropoulou et al., 2014; Raak et al., 2017). Recovering and recycling appear more successful for waste reduction (Eriksson et al., 2015). More precisely, food recovery is related to the distribution and possible human re-use of food surplus which has not yet become waste. The recycling strategy, instead, is referred to the excess of food already become waste (that can no longer be reused by people), which can be employed in the production of other goods and services, such as animal feed or compost, or as an energy source, or for industrial needs, etc. (Mourad, 2016). In this way, the final product of a production chain becomes the starting point of another transformation process (Figure 1.1).

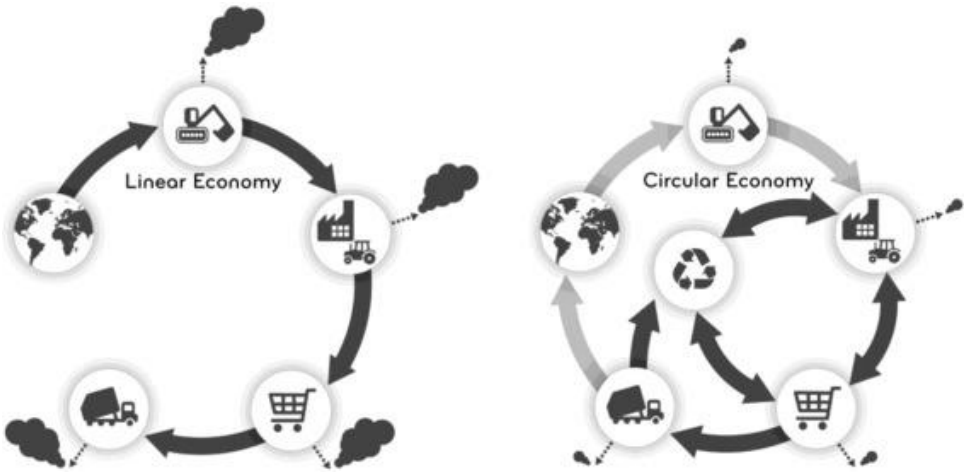

Figure 1.1 The difference between a linear (left) and circular (right) economy model. The linear economy is characterized by a beginning (resources extraction and consumption) and an end (waste disposal and pollution). In a circular economical pattern, re-utilisation of resources replaces the waste destruction, reducing environmental impact and wastes. (Sauvé et al., 2016)

Recycling is precisely what allows the transformation of the food production system into a cycle. In our difficult economic context, characterized by strong competitiveness, food companies are increasingly interested in the re-use of natural resources, and large investments are being made in this type of prevention activities. However, the transition towards a circular economy is not without difficulties. Many authors have identified and studied the obstacles in the adoption of a circular and sustainable food production strategy. According to the most recent studies, more appropriate economic and policy frameworks, improved infrastructures, and precise data on the available amount of food waste are necessary to help companies in the transition from the traditional linear economy model to solutions that support sustainable development (Araujo Galvão et al., 2018; Kirchherr et al., 2017; Ritzén & Sandström, 2017). The compositional heterogeneity and the rapid degradability of the leftovers are also problematic aspects of the agri-food waste control path. The best-known approach used in waste control is the direct one. Fuels, heat, power, and chemicals are directly “extracted” from materials that would not otherwise be used (Cherubini, 2010; Cristóbal et al., 2018). Developing an adequate food waste direct biorefinery process requires it to be adapted to the quantity, the type of food wastes, and the final product to be obtained. If well adopted, biorefineries’ potential added-value

products and chemicals from food waste biomasses could be one of the most important points in the “resource revolution” (Cristóbal et al., 2018; Heck et al., 2014; Matharu et al., 2016). Besides fuel and energy, valorized products production opportunities through a direct food waste biorefinery was already evaluated by many authors in literature (Dávila et al., 2017; Lohrasbi et al., 2010). Just to name few prototypical examples, the investigation of Davila et al. 2015, for example, concerned the production of pectin and other chemicals from citrus peels (Dávila et al., 2015); Hernandez et al. 2014 reported about olive leftovers biorefinery to obtain antioxidant potential (Hernández et al., 2014); Shahzad et al. 2017 explored the possibility of extracting biopolymers from animal processing wastes. (Shahzad et al., 2017). To date, interest in waste biorefinery is growing, and different techniques and patterns are emerging in order to avoid agro-food waste losses, thereby getting enhanced products or biomasses (Wellisch et al., 2010). Among the most innovative approaches in food residual biotransformation, is the use of biological systems such as algae, bacteria, insects etc. These macro- and microorganisms grow and live on the organic waste by using the low value residual substances in the biomass, facilitating the formation and the extraction of valorized elements (Rajak et al., 2020). If developed under the right conditions, the result is a totally new product: a uniform and enhanced biomass to be applied as starting material in a large range of industrial sectors. This indirect solution guarantees significant environmental and economic benefits. Heterotrophic microalgae, for instance, are reported to affect positively the air quality and convert organic waste in a good and cost effecting protein and lipid source (Smetana, 2020). Bacterial fermentation is known as a way to obtain energy and biologically active compounds from low value by products (Sabater et al., 2020; Yasin et al., 2013). With the aim of controlling and converting residual organic biomass and improving the impact from both an environmental and economic viewpoint, insects implication is even more advantageous (Smetana, 2020).

1.1.4 Insect as waste converters into new products

A so-called “selective breeding” of insects, viz., controlled by man, so as to bio convert specific food wastes, is economically helpful in terms of time and efficiency (Jensen et al.,

2017). Although rapidly expanding, using insects as a waste management tool is still a young field in a preliminary testing phase. In recent decades, industrial insect production, with an emphasis on developed insect breeding methods, is increasingly attracting funds and interest from new companies on a global scale (Fowles & Nansen, 2019). Obtaining valorized secondary products with a high economic value by acquiring and using cheap raw materials (food waste) and simply setting insect breeding is a very attractive business idea for many investors. Biodiesel (defatted insect meal to be used as protein food and feed) pharmaceuticals are the products mainly obtained by refining the insect biomass after the bioconversion process. The common way to produce biodiesel is by processing oilseeds. Notwithstanding, the exclusive use of this raw material has a significant impact on arable land availability and the economic sector. In specimens such as black soldier fly, it can extract and use the fat accumulated in the larval body during waste bioconversion as a perfect alternative to produce non-fossil fuels. Obviously, biodiesel extraction is higher if insect bioconversion occurs on fat-rich food wastes: oil extraction is maximized by managing the waste problem at the same time (Fowles & Nansen, 2019). Natural fertilizers are instead the principal commodity derived from frass, which is obtained by separating the insect biomass from the substrate used for larval growth. It was observed that the composition of the organic biomasses after the insect development was nearly equal to that one of artificial fertilizers, with one advantage over higher pathogenic microbes and pesticides control. (Lalander et al., 2016; Salomone et al., 2017). Yet, it should be noted that these are not the only high-value products that may be derived from the insect bioconversion chain. The second half of the XX century saw the growing interest in using insect biomass (from bioconversion) for the feed sector and human consumption. Around 70% of food products consumed by the increasing world population is expected to be of animal origin (Godfray et al., 2010). In this frame, the world is carefully concentrated on the need to find an alternative eco-friendly solution to satisfy global protein requirements without increasing the common livestock farming. Insect farming for organic waste control can also solve this problem (van Huis et al., 2013). Several studies have been conducted on insect protein fraction (Zielińska et al., 2017). With a protein amount ranging from around

30% and 70% and very good balance in essential amino acids, insects are considered a high biological value protein source (Finke, 2013). Moreover, the development speed and the feed conversion ratio are very high if compared to common livestock. In this perspective, black soldier fly is one of the most promising and studied species, together with mealworms and crickets. In particular, the final weight of black soldier fly larval biomass feed with 100 kg of food waste can be doubled or, even more, compared to that one obtained for other animals (chickens or beef) (Fowles & Nansen, 2019). Nowadays, even if agencies as Food Authority Organization are trying to increase public awareness of possibility to consume insects as food, other organizations such as EFSA underline the risks and need to lay down rigorous legislation before allowing human consumption (EFSA, 2015; FAO, 2017). To date, just two species, dried *Tenebrio molitor* (Regulation (EU) 2021/882), *Locusta migratoria* (Regulation (UE) 2021/1975), have been obtained the authorisation to be commercialized as food and a third, *Acheta domesticus*, is under consideration.

The high-quality insect protein fraction has instead found the main application in the animal feed sector, where it was still necessary to find alternative sources of support to make up for the agricultural resources scarcity. Nevertheless, also here there are still limitations. To date, there are only seven species authorized to be reared for feed production: *Hermetia illucens; Musca domestica; Tenebrio molitor; Alphitobius diaperinus; Acheta domesticus; Gryllodes sigillatus; Gryllus assimilis* (EU Regulation 2017/893). From 2021 it is authorized the use of insect proteins just in pet, fish, poultry, and pig feed. However, the legislation is expected to be revised in the coming years and it is expected that by 2030, over 730,000 tonnes of feed will be produced globally from large-scale insect farms, and that around 10% of the EU rate of the total protein supply for both food and feed will be represented by insect proteins (Gasco et al., 2020).

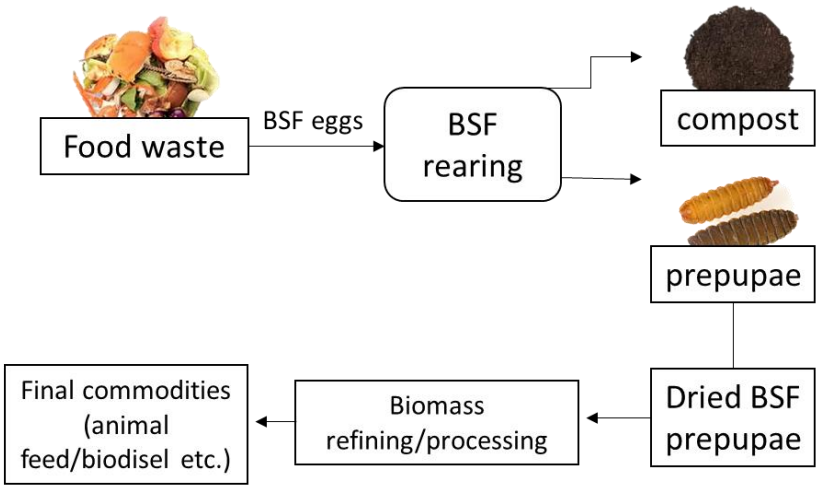

Figure 1.2 Black soldier fly bioconversion of organic wastes into valuable products. Image readapted from (Surendra et al., 2016)

1.2 The role of insects in the new circular economy concept

1.2.1 The importance of an adequate rearing substrate association with insect species

A controlled insect-based bioconversion could solve both the decrease of the food waste and the increasing need for alternative sources of protein, biofuels, and fertilizers (FAO, 2017; Parfitt et al., 2010). As a result, the idea of exploiting the natural ability of some insect species in growing on residual leftovers and bio converting them as a sustainable solution to manage and reduce food wastes, attracted the interest of the industrial sector in developing controlled breeding models and techniques. Nonetheless, the bioconversion of organic material through insects is not as simple as it could appear. First, not all insect species are equal: because of the morphological and physiological differences, some species, including black soldier fly (BSF, *Hermetia illucens*), are more suited to the role of bio converters of organic waste than others. Moreover, each bio converting insect species have specific substrates they prefer to use, and which encourage their growth. This is because organic wastes are compositionally very different from each other, and each insect can differ in nutrient needs, which cannot be provided by all the leftovers in the same way. Just to give an example, as reported by some authors, a low protein organic waste is not

an appropriate substrate of growth for housefly larvae, while it is enough for the nutritional requirements of species as Black soldier fly and mealworm larvae (Hogsette, 1992; Li et al., 2013; Manurung et al., 2016). This last species, preferring dried substrates, does not grow well on wet wastes, where the moisture content is instead perfect for the requirements of Black soldier fly and housefly larvae (Cheng et al., 2017). High yield, cost-effectiveness and low environmental impact of the process are clearly the advantages of using insects to manage the excess of organic waste generated. Adapting inappropriate wastes to insect needs (for instance, drying a wet substrate) generates operating costs and resource consumptions that would jeopardize the attainment of the objectives. Thus, when insects are involved in a bioconversion mechanism, it is essential to make sure that insect species and food residuals are compatible with successful waste reducing and recycling (Fowles & Nansen, 2019). To date, just a few insect species were tested and used in the bioconversion process. Fowles and Nansen (2019) reported a list of insect species (for example *Hermetia illucens; Cydia pomonella; Teleogryllus testaceus; Tenebrio molitor*) with their corresponding ideal waste pairings and the bioconversion output. Also, Leni et al. (2021) summarized the most compatible agro-food by products for some of the most important bio converter species. According to what observed by these and other studies, species as *Hermetia illucens* (Black soldier fly), *Tenebrio Molitor (*yellow mealworm) and *Alphitobius diaperinus* (lesser mealworm), crickets and silkworm are very promising species for bioconversion of wastes. However, crickets and silkworm are more selective about the substrate. *Tenebrio molitor* is more flexible, can be adapted to different wastes, and its molecular composition seems to be affected by the matrix (Chieco et al., 2019; Ravzanaadii et al., 2012). As above-mentioned, genus mealworm in general still prefers dry substrates, like cereal by-products. This excludes wet substrates from the list of easily convertible wastes by these insects' species. Because of these problems, in the literature, the studies on waste bioconversion using mealworms as a tool of recycling are limited. Consequently, also the investigations related to the effect of the substrate on their nutritional composition are scarce (Leni et al., 2021). More in general, all the insects present on the planet are organisms that feed on organic detritus; therefore, all species are potential bio

converters. Further studies to maximize the potential of insects already examined and to learn more about the bioconversion ability of other species not yet considered, are urgently needed to extend the benefits of this novel market and ensure a more sustainable present. For instance, insect species belonging to the Termitidae family were observed by Varelas and Langton (2017) to easily grow on lignocellulosic substrates as wood industry residuals (Varelas & Langton, 2017). More experiments, e.g., on their ability to obtain nutrients by digesting forest organic waste bio converting them into secondary valuable products such as biofuel and compost, would help to reduce the high annual build-up of lignocellulosic waste and provide more environmental protection opportunities.

Today, black soldier fly is the most studied insect species concerning the bioconversion of organic waste. What clearly emerged from the studies of Fowles and Nansen (2019) and Leni et al. (2021) is the greatest ability of Black soldier fly in growing on an extended variety of organic wastes and consequently its large use, compared to other species, for high value biomass production.

1.2.2 Black soldier fly potential in bioconversion of organic materials

Black soldier fly can use as a growing substrate a large range of industrial food residuals such as cereals, vegetables, fruit, meat, legumes, etc., also when these matrices have a very low nutritional value as with plant-based waste. This highlights the great adaptability to different growth environments (Leni et al., 2021). As observed by Bortolini et al. (2020) and Shumo et al. (2019), black soldier fly can also develop on matrices not yet allowed by EU to be used as substrates for bioconversion processes (Bortolini et al., 2020; Shumo et al., 2019). Notwithstanding, good development and adaptive capacity skills on all organic substrates do not necessarily result in a maximum bioconversion and growth yield. For instance, the Black soldier fly can still develop on nutritionally poor substrates, e.g., vegetable by-products, without maximizing bioconversion yield due to the limited nutrients supply. In this situation, the role of Black soldier fly as a perfect tool of waste valorization is reduced, and the principle of sustainability diminishes. So, over the last few years, studies on black soldier fly farming and on its correlation with food wastes' features, such as nutritional composition, seasonality, combination, food chain stage, treatments (etc.),

have been intensified to ensure an insect breeding continuity, optimizing its final performances and yield of secondary products (oils, fertilizers, feed, pharmaceuticals). In this context, composition in water, lipid, protein, carbohydrate, of the growing substrates is the most important factor to take into consideration. High moisture content, for example, was found to be most important for the fast development of the black soldier fly species (Cammack & Tomberlin, 2017). However, there are still few studies where the effects of the macromolecular composition of the substrates on the growth of the insect are evaluated. These investigations should precede bioconversion processes, to evaluate the best insect-waste pairings and the best use of the insect resource. As a whole, there is a real potential for producing black soldier fly, as they need low resources producing in return high added value products. Nevertheless, this is still a new field of waste management and, although black soldier fly is one of the most studied and promising species in the leftover's bioconversion, a lot of aspects related to its nutritional needs are still to be learned in order to improve the process performance.

1.2.3 Authorized and unauthorized substrates for insect rearing

Food residuals, manure, mixtures of municipal organic and/or catering waste, animal by-products are all substrates easily degraded by many species of insects. In particular, the Black soldier fly is able to use equally all these kinds of discards as a growth medium, proving to be an exceptional waste volume reduction tool (Bortolini et al., 2020; Leni et al., 2021). E.g., a good ability to grow on manure was observed in *Hermetia illucens* by Gold et al. (2018) and others, with the additional benefit of generating a valorized biomass in terms of protein and lipid content (Gold et al., 2018). Since the European Union Regulation 1069/2009 reports that insects are farming animals, no different from common livestock, they must respect specific breeding rules (Leni et al., 2021). Thus, edible insects are not allowed to grow on many organic residuals, even when insect breeding is finalized to the reach of non-feed/non-food goods.

Indications on the substrates that can be used for rearing the seven insect species approved for feed purposes are reported in the Regulation (EU) 2017/893 of 24 May (European Commission, 2017). For instance, former foodstuff containing vegetables, eggs, milk, or

milk derived products, fishmeal and all vegetable products are allowed. All other products of animal origin not cited above are instead forbidden. Manure and human food waste (food and kitchen waste) are also not included in the list of authorized growth medium. The reason for all these restrictions derives from the fact that according to the European Commission, not all the rearing substrates derived from organic waste products can be considered safe. The potential presence of chemical (such as heavy metals or additives), physical (such as plastic residuals) or biological (antibiotics, prions) contaminants in the substrates and microbial risks (pathogens) are all aspects to be considered in evaluating the adequacy of wastes adopted as a growing insect matrix, especially in the case of a distribution of final outputs in feed and food sectors. The use of catering waste or food containing meat or fish residuals for example as insect feedstock can be dangerous precisely because of the accumulation of contaminating substances and the microbiological risk, including prions. For the same reason, EU authorized insect feed does not include manure for insect farming (Bortolini et al., 2020). Interestingly, some insects like the black soldier fly species can release into the growing matrix antimicrobial compounds able to control hazardous pathogens potentially present in this kind of organic wastes, “sanitizing” the substrate (Fowles & Nansen, 2019). In this regard, black soldier fly has significant but still unexplored potential. Furthermore, the ban on insect breeding on certain types of organic waste is more preventive than a necessary measure. In fact, there are not many studies in the literature that point to a real danger in using manure or other kinds of forbidden organic wastes as insect growing substrates for food and feed ingredients production. Further research is needed to investigate the safety aspects of this system. A proof that the use of wastes considered unsafe in fact does not affect the quality of the biomass resulting from bioconversion, would allow both sustainable control of other wastes difficult to manage and the possibility to test more insect-waste combinations with the goal of maximizing the breeding performances to obtain secondary valorized products. Hopefully, if legislation were revised, it will be possible to transform this opportunity into real results. Globally, the main issues that emerged from this discussion are the need to reduce management costs and environmental impact derived from the waste excess from

the food supply chain and the demand to find low cost and abundant alternative food and feed sources. Insects' bioconversion can be a part of the solution for these issues, but there are still some open questions, e.g., the need for more in-depth studies on the effects that different substrates have on the nutritional profile of the final insect biomass, and the necessity of testing different substrates or mix to understand how to maximize the breeding performances and outputs of insect-based bioconversion process. In **Chapter 2** of the present PhD thesis, the potential of Black soldier fly prepupae reared on vegetable leftovers with different seasonality was exploited to the obtainment of compounds with high added value and further industrial and agronomic uses such as food/feed, soil improver or fuel. Optimized leftover combinations in terms of nutrients were tested as insect rearing substrates, to assess the mix able to offer the best wide range of performance capabilities in terms of insect growth and nutritional composition, with a focus on the protein and amino acid content of Black soldier fly prepupae.

1.2.4 Nutritional composition of Black soldier fly

As many studies reported, nutritional composition of insects depends on several factors. Some factors are external, e.g., growing environment or rearing substrate etc. Others are related to the species, life stage, metabolism (Gasco et al., 2020). However, insects generally present a very high (and diverse) protein content with a high biological value, commonly ranging between 30 and 70% on dry weight (Finke, 2013). A high and equally diverse fat content (from 10 to 30%), with variable fatty acid composition, also characterizes the nutritional profile of most of the insect species. They can be considered a good source of minerals as well, especially iron and zinc, and vitamins like vitamin B12. The complete nutritional profile makes them particularly adequate for animal and human nutrition. In addition, by modulating the substrates used for their growth, it is possible to obtain a nutritional profile more adapt to specific animal needs. For instance, it was observed that an enriched polyunsaturated fatty acid or mineral insect biomass can be obtained simply by using rearing substrates naturally high in these compounds (Gasco et al., 2020). Black soldier fly is not only one of the most efficient species in waste bioconversion process. It is also one of the most promising as an alternative food/feed and

protein source. Moreover, it is one of the most able in enriching its nutritional composition according to the nutrients present in the diet (Barroso et al., 2017). The nutritional value of black soldier fly larvae, the development stage of greater interest for feed and food production, has been widely discussed in literature. Depending on the quality and quantity of feed consumed, black soldier fly larvae can present a variable protein and lipid content. The protein and lipid amounts can range respectively from 37 to 63% and 7 to 39% on dry basis (Barragan-Fonseca et al., 2017). On the contrary, the profile of amino acids has been found quite similar among the various studies. BSF larval protein content is particularly rich in lysine, and generally methionine, histidine, and tryptophan content are higher if compared with the aminoacidic profile of soybean. The fatty acid profile, always with variations depending on the diet, is particularly rich of saturated fatty acids such as lauric, palmitic and stearic acid. Unsaturated (mono and poly) fatty acids, except for oleic acid, are instead present in a less percentage (19-40%), but still considerable (Surendra et al., 2016). The mineral content appears to be higher in black soldier fly compared with other edible insect species. Total ashes, ranging between 9 and 28% (Finke, 2013), with amounts higher in the larval stage with respect to the other life phases. Specifically, manganese, iron, zinc, copper, phosphorus, and especially calcium (concentrated in the chitinous shell) were found in higher concentrations. Finally, black soldier fly larvae were found to be a good source of the polysaccharide chitin. This biopolymer is part of the insect exoskeleton and Its content in the black soldier fly larval body is estimated to be around 9% (Caligiani et al., 2018).

1.2.5 Insect chitin and its potentialities

Chitin is the second most widespread structural polysaccharide after cellulose and absolutely the most abundant amino polysaccharide present in nature. It consists of single repeated units of the amino sugar N-acetyl-D-glucosamine, which are joined to form a linear chain through a condensation reaction by a specific membrane-integral glycosyltransferases (chitin synthase).

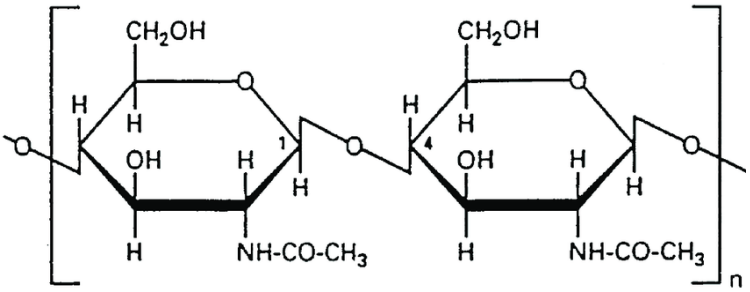

Figure 1.3 chemical structure of chitin

Following the polymerisation reaction, the crystalline microfibrils deriving from the natural reorganization of the individual polymers, can form more complex structures with other macromolecules such as sugars, minerals, proteins (glycoproteins, proteoglycan). These are the principal parts of the cell walls of fungi and nematodes and of the protective structures of arthropods such as insects and crustaceans. As part of the cuticular exoskeleton and some internal structures and for its good strength characteristics, in arthropods, in particular insects, the role of chitin is essential for development and protection of the fragile components of the body (Merzendorfer, 2006). In recent years, the economic value of chitin and its derivates is growing considerably thanks to the innumerable potentialities of applications that this biopolymer can offer to medicine, food, textile, cosmetics sectors (Caligiani et al., 2018). In figure 1.4 the main properties and applications of chitin and chitosan, its deacetylated form, are reported. Additionally, chitin is a natural biopolymer that responds to the need to use renewable resources reducing the use of virgin raw materials to obtain valorized secondary products. It is estimated that every year about 10^{11}-10^{14} tons of chitin is produced by a great number of organisms. Consequently, a significant quantity of chitin is obtained as waste product from crustaceans, molluscs, insects, and fungi food processing industry (Kim, 2010). Indeed, crustacean waste from food industry (body shells) and fungal mycelia were the main source of chitin for secondary industrial purposes. In the recent context of insect introduction in food and feed, a new high-potential chitin source, to be used for their biological and physicochemical properties, could be on the market.

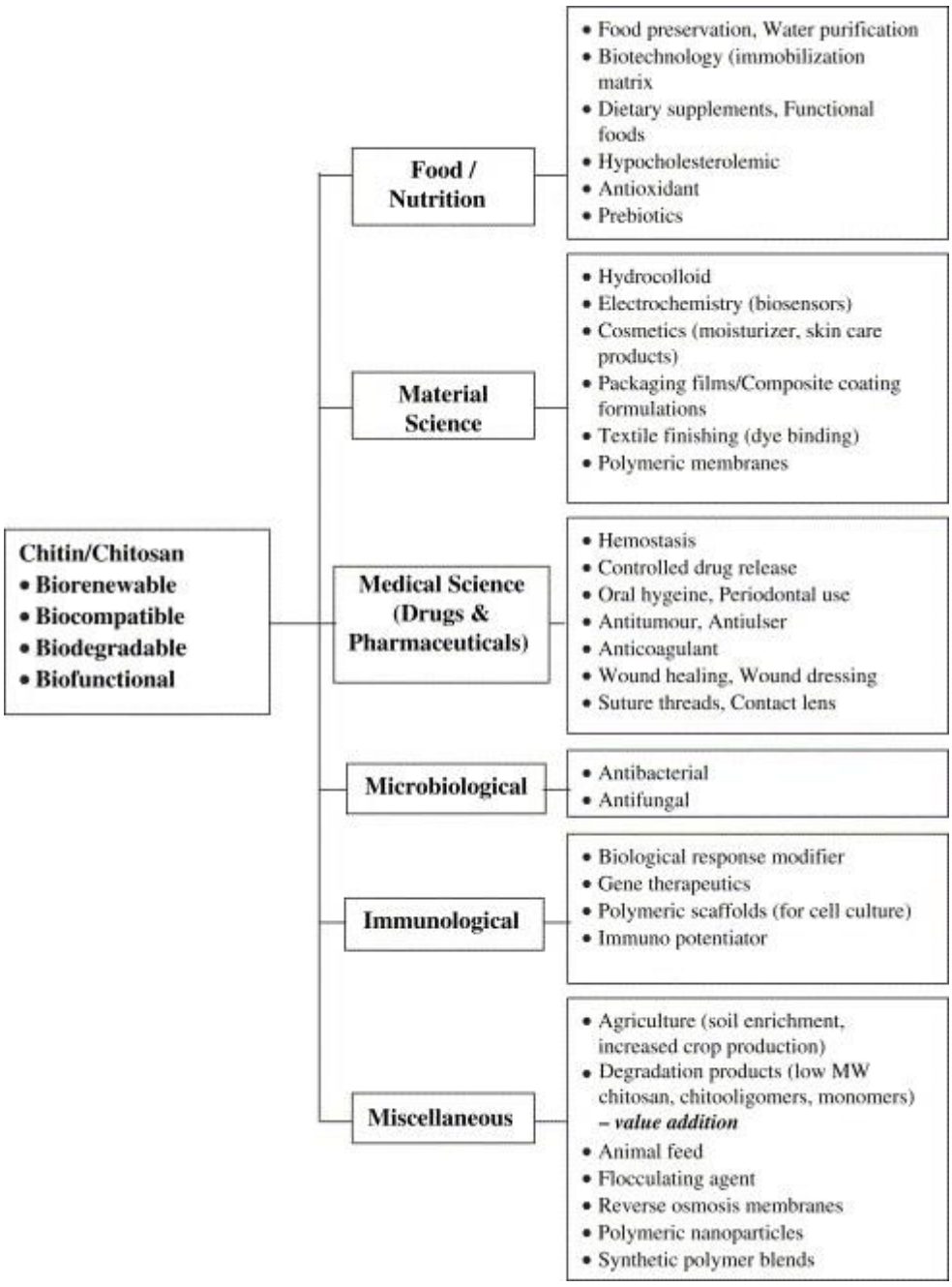

Figure 1.4 Main properties and possible applications of chitin and chitosan. "Reprinted from Publication Trends in food science & technology, 18/3, Prashanth, K. H., & Tharanathan, R. N.Author(s), Chitin/chitosan: modifications and their unlimited application potential—an overview, 117-131, Copyright (2007), with permission from Elsevier".

1.2.6 Chitin quantification challenge

In insects, chitin is present in many structures, but most is found in the cuticular one. As reported by many authors, extracting chitin from the body of the insect requires multiple steps. At first, it is essential to remove lipid and protein fractions from the insect matrix. In this regard, Leni et al. (2021) reported in detail about the most common lipid and protein extraction methods. After that, the resulting substrate must follow a demineralization protocol to eventually obtain pure chitin. Generally, it is carried out through acid hydrolysis with HCl 2M for 24h (Leni et al., 2021). Extracting chitin from insects to be applied in industrial sectors as a renewable resource is a long but simple process that does not require special equipment or experience. The true challenge, when dealing with insect chitin, as with other biological substrates, is to determine its accurate content in the body structures. As already discussed, when insects are reared with the aim of achieving valued biomass to be used in the feed and food market, it is essential to know their whole nutritional composition to adequately satisfy the dietary requirements of the livestock and humans. Several data are present in the literature on the nutritional composition of edible insects. Standard procedures are generally applied for the determination of their protein, lipid, ash, and moisture content. As it regards accurate chitin quantification, the situation is more complicated, principally due to the bonds it forms with other macromolecules present in the biological matrices and for their low solubility in almost all the solvents (Han & Heinonen, 2021). The main chitin determination methods utilized require simply to weight the biopolymer collected after alkaline treatment (gravimetric method). Its structure, in fact, resistant to this kind of treatment, is the only one expected to maintain insolubility with respect to the other biopolymers (Tsurkan et al., 2021). However, hydrolysis conditions need to be well controlled. If too strong they can cause the deacetylation of chitin which loses the characteristic of insolubility, creating big limitations to this technique. Until recently, insect chitin was generally measured as acid detergent fiber (ADF) fraction, as proposed by Finke (2007/2013) (Finke, 2007, 2013). For the large number of amino acids dragged into this fraction, however, an inaccurate result was achieved.

Alternative chitin determination methods developed during the years are numerous, but, despite all the scientific research on chitin identification, all these methods remain inaccurate. Tsurkan et al. (2021) reviewed the current state of the art of the methods of chitin identification, highlighting the precision by which in the literature are collected information on biosynthesis, distribution, characterization, properties, and applications of chitin with respect with its quantification. Additionally, it is also provided a list of limitations for each method. Direct methods, such as structural (i.e., X-ray diffraction and scattering) and spectroscopic methods (IR, Raman, and Near-edge X-ray absorption fine structure spectroscopic method), often require high amount of sample and generally are more intended to identify the different molecular forms of chitin present in the matrix rather than to quantify it. Among the spectroscopic methods the Infrared spectroscopy and Nuclear magnetic resonance spectroscopy NMR are known to allow a good identification of chitin in biological samples. The first is mostly used to characterize the degree of acetylation of chitin and chitosan and the second one is not widely used for the need to dissolve the sample in concentrated acids (deuterated hydrochloric acid) which can destroy the chitin structure (1H NMR) or to purify chitin from the other molecules to obtain good results (Solid-state 13C NMR spectroscopy). Given the difficulty in analysing chitin directly, a lot of methods are based on the indirect detection of the polymer. Strong acidic hydrolysis can break the bonds of chitin, generating free N-acetyl-glucosamine or glucosamine, more easily measured. For monomer identification colorimetric assays and chromatographic and electrophoretic methods are generally applied. Most colorimetric methods used for glucosamine quantification are very old (DISCHE & BORENFREUND, 1950; Elson & Morgan, 1933) and in addition, they require high analyte concentrations. The best-known electrophoretic method in the glucosamine quantification is the High perform capillary electrophoresis (HPCE), with the limitation to be an excessively sensitive technique to various chemical compounds in a biological sample that can prevent glucosamine specific signal recognition. For this reason, a purification of the chitin fraction before the analysis is advisable. As a whole, among all indirect approaches, the chromatographic ones are considered more specific in the quantification of glucosamine in

biological samples, even if they need many samples preparation steps, such as a sample clean-up by using acids, which require long times of analysis combined with a lot of chemicals reagents consumption (D'Hondt et al., 2020). Biochemical and immunochemical methods, e.g., the chitinase test, are also used in chitin identification but they are very expensive and laborious techniques (Tsurkan et al., 2021). Globally, Tsurkan et al. (2021) evidenced the great difficulty of all these methods in analysing crude biological samples, where the presence of other chemical compounds interferes with correct quantification of the chitin fraction. The increasing economic interest in the black soldier fly larvae highlighted the need to develop a more efficient method in the quantification of chitin in its matrix. Therefore, in the recent years, a lot of studies have been done and efforts have been made to improve the procedures and to shorten analysis times for insect chitin quantification. Moreover, an important purpose is also to develop a method which reduce the interference between chitin and protein fraction. Insect chitin, in fact, at the level of the exoskeleton is used to form complexes with other molecules such as cuticular proteins, making difficult its direct quantification. In addition, the amino group present in its structure also makes inaccurate the analyses related to the insect protein fraction, which becomes regularly overestimated when standard methods are applied (Kjeldahl). In these terms, only few colorimetric and chromatographic methods recently developed can be considered efficient in the quantification of chitin in insects (D'Hondt et al., 2020; Han & Heinonen, 2021; Katano et al., 2016). In edible insects it is essential to define the chitin content, also due to the negative effects it could have on food texture or, once ingested, on intestinal nutrient absorption (Shahidi & Abuzaytoun, 2005). In fact, apart from all its positive features (biodegradability, biocompatibility, non-toxicity, potential prebiotic, antimicrobial activity), it is known that chitin could have anti-nutritional effects. An excess of chitin concentration in feed can be counterproductive and can be better controlled having a method for its accurate quantification (Hahn et al., 2018). Further studies are needed to suggest alternatives to the techniques already developed and enrich the literature with rapid, economic and simple methods for assessing chitin present in insect biomass. It would also be interesting to focus on research of methods that are able to

quantify at the same time different insect macromolecules, halving the compositional analysis time. In **Chapter 3** of the present thesis, a new RP-UPLC-ESI/MS method that meets these requirements is presented. This analytical approach has been validated for simultaneous assessing chitin (as glucosamine) and protein (as total amino acids) content in insects, with a particular focus on the BSF species.

1.2.7 Large-scale insect production

Bioconversion insect-derived products have a very high economic value because they resulted from of low-cost by products. That is why the edible insect rearing is a growing industry. All the benefit derived from the ability to control insect growth increased the industries and companies' interest in investing in this field, accelerating the transition from studies on a laboratory scale (to evaluate perfect insect-waste pairings) to industrial scale experiment, and consequently to the present time where insect mass production for business purposes is in some cases a reality (Huynh et al., 2021). An adequate quantity and type of food waste were seen to be, in small scale studies, the most important factor for insect rearing cost-effective and successful in terms of valuable outputs. The same, however, does not hold for mass-reared insect species. To be globally advantageous, both in terms of product quality and quantity, costs and environmental impact, a large-scale insect production implies a consideration of many third-party factors that can negatively affect the process. More specifically, elements such as lighting, sanitization, environment, and insect derived waste management, special spaces for egg position etc. need to be optimized for a sustainable system. The main risk in fact, in insect mass rearing, is that trying to control waste could subsequently trigger other losses. Moreover, the high economic value of the bioconversion resulting biomass on the market could bring insect producer companies to push on production, forgetting the real aim of the insect-based rearing, that sustains a zero-waste system (Huynh et al., 2021). As Chavez (2021) rightly pointed, a "zero waste" approach provides for the recycle and reuse of the largest number of by-products, including those resulting from the same bioconversion process such as insect derived wastes. These waste as well can generate an environmental impact, if not managed adequately.

1.2.8 Insect bioconversion process wastes

Scientific literature is rich in studies where the ability of insects, especially black soldier fly species, to digest a large range of organic wastes, also those ones not yet permitted to be used as breeding substrate by European legislation, is shown. On the contrary, it suffers from a lack of information on the type and relative amount of waste generated by the process and the likely significant environmental effects they may have if produced in large quantities (Guo et al., 2021). Among the few life cycle assessment (LCA) studies of insect rearing conducted, methane (CH_4), nitrous oxide (N_2O) and other gas emission data, global warming potential (GWP), environmental benefits of breeding insects compared to normal livestock, are the aspects commonly monitored in terms of sustainability (Mertenat et al., 2019; Smetana et al., 2016). Many other elements related to the bioconversion process, nonetheless, should be measured to completely assess the life cycle impact of a systemized insects' implant. To keep an insect's industrial scale bioconversion going, all the stages of the insect life cycle (egg, larva, adult etc.) should be present. Focusing on the life cycle of black soldier fly, for instance, it varies from 5 to 21 days (Nakamura, 2011). In breeding-controlled systems, some specimens at the pre-pupal stage are kept aside and once adults used for the mating ritual. From each gestation, generally, the female flies lay between 400-900 eggs. After around three days, the eggs hatch. In adequate feeding conditions, insects rapidly cross the five larval stages, finally arriving at the prepupa stage. This insect stadium differs from the previous one for the exoskeleton pigmentation and hardening (sclerotization). The transition from pre-pupal to pupal phase normally takes about 7 days. From this moment, most of the pre-pupal biomass will be used for all potentially advantageous applications already cited. A smaller part will follow the metamorphosis to adult stage to allow continuous system operation.

Maintaining active the black soldier fly's life cycle obviously generates insect derived wastes. During the metamorphosis from prepupal stage to imago of black soldier fly, the insect protective shells, also called puparia, are delated, becoming an important system leftover. Also the adult flies' bodies, once completed their life cycle, can be considered as products that should be discarded. The potentialities in reducing the environmental

pressures, producing valuable products of industrial interest, and being an alternative food and feed solution have contributed to an exponential and sudden increase in insect large scale production. Industrial scale rearing and high reproduction rates can also generate an excess of prepupae which cannot found immediate application in the industrial sectors. This biomass surplus may also be considered as an element to be managed, because, in the presence of massive insect production, these insect residual materials can represent a significant problem. At the best of our knowledge, to date, there is not an objective evaluation in terms of type/volume of wastes directly generated by the insects themselves in a bioconversion process or their management/re-use.

1.2.9 Fermentation as valorization technique for insect-related wastes

When fermentation take place on a solid and relatively humid substrate, it is named solid state fermentation (SSF) (Pandey, 1992). This technique has its historical roots in Asian culture, where already in ancient times it was used to positively modify foods and obtain bioactive compounds. For instance, in China it was used to obtain local fermented food, cheese, koji (fermented rice), all known to be added value products. During the time SSF has increasingly been explored and used all over the world to obtain valorized food, with positive effect on living beings' health. Soy, milk and derivates, grains, vegetables are just a few examples of food products that acquire benefits following fermentation process. It is established that the regular consumption of fermented foods improve intestinal tract health, immune system reactions and bioavailability of nutrients, reduce intolerance symptoms as for lactose, etc. (Hasan et al., 2014). But the fermentation positive effects are not only health related. It is known that fermentation increase food shelf life, improve food digestibility, prevent toxic effects, enhance flavour, taste and aroma of foods and their nutritional properties. On this matter, Hasan et al. (2014) explain how microorganisms involved in a fermentation as well as having a catabolic behaviour, can synthetize compounds such as vitamins or to release nutrients from indigestible structures for humans such as from cellulose, enriching the nutrient profile of food. All effects on both health and food technical aspects are related to the influence of the microorganisms involved in the process on food pH, the ability to contrast pathogens, to produce enzymes etc. Among all

the microorganisms, Lactic acid bacteria (LAB) are the most commonly used for food fermentation. This group includes *Leuconostoc, Streptococcus, Lactobacillus, Enterococcus, Aerococcus* and *Pediococcus* species (Moslehi-Jenabian et al., 2010). Most positive effects of fermented products were proven to derive from lactic acid fermentation (Hasan et al., 2014). Nowadays, the research field is working towards the evaluation of ever-different combinations of microorganisms, food and working parameters, to improve the nutritional value and the palatability of a larger range of products, including novel foods as insects. Eating insects still remains a taboo in most Western cultures, especially for the disgust to their shape, taste and flavour (Belluco et al., 2015). In this regard, studies have also been carried out on the possibility to improve the acceptability in terms of sensory features and simultaneously the nutritional, technological and bioactive properties of insects, by using fermentation technique (Castro-López et al., 2020). Even if research is limited, the results of an insect-based fermentation are clear. Not only it was observed that following fermentation practice insects acquired higher sensory qualities, appearing both more acceptable and preferred as food and feed. Some studies conducted on the possibility to obtain bioactive compounds by fermenting edible insects with LAB reported the presence of antimicrobial activity in the matrix resulted by the process (Hadj Saadoun et al., 2020; Kewuyemi et al., 2020). Along these lines, other biological activities were recognised in fermented edible insects: Cho et al. (2019) have been observed the presence of tumour-suppressive bioactive components in *Bombyx mori* larvae after *Aspergillus kawachii* SSF (Cho et al., 2019). High antioxidant activity was confirmed by Jang et al. (2018) in B. subtilis fermented *Tenebrio molitor* larvae (Jang et al., 2018). Enhanced nutritional profiles of edible insects have also been observed (Castro-López et al., 2020).

Clearly, solid state fermentation has the great potential to improve the positive insect products characteristics, including nutritional value and functionality. Nonetheless, microorganism behaviour on this novel food is not still enough explored. Numerous combinations should be tested, and many variables should be accounted for to have a complete picture of the effect of fermentation on insect matrix. Currently, the promising

results obtained encourage to move forward new studies, expanding the range of investigation.

SSF of chitin rich by-products was already investigated and the results obtained are very promising in terms of valuable compounds. For instance, by products derived from the industrial crustacean processing (cephalothorax and carapace) were used as substrate of SSF obtaining enzymes like chitinase and chitosanase, with great interest for biomedical, food and agrochemical industry (Nidheesh et al., 2015; Suresh & Anil Kumar, 2012). Insect-derived wastes from bioconversion processes (puparia and dead adult flies) have many compositional features in common with the scraps of crustacean processing industry. This opens positive prospects on the use of SSF to valorize insect derived by-products as well.

Considering the fast increase in insect industry, it is likely that the number of by-products derived from the high insect production will increase accordingly, constituting an important issue. Considering valorization effects that SSF has on organic products (including edible insects) and by-products, the use of insect leftovers as substrate for microbial growth can be considered as a perfect way to manage and valorize these kinds of residuals. The need to manage insect waste is a relatively recent problem. This is one of the main reasons why the literature is poor of studies related to insect by-products valorization techniques, such as their use as fermentation medium. In **Chapter 4**, the possibility of enhancing and transforming black soldier fly by-products in sources of bioactive compounds through solid state fermentation was evaluated. The nutritional value of insects-derived wastes before and after fermentation was accurately investigated and all the differences (both among the strains and substrates) were well estimated.

1.2.10 Insects as alternative source of valuable (and bioactive?) protein

As reported by Van Huis et al. (2013), insect proteins are one of the possible solutions to the increasing world protein demand, for their high biological value and for their low production costs (van Huis et al., 2013). The protein content and amino acids composition of the most of edible insects was found to be comparable to that of high biological value products, such as eggs and soybeans (Leni et al., 2021; Payne et al., 2016) Considered the

recommended protein daily requirement for a human adult, in some cases it is also exceeded with just 100g of insects' dry product (Food and Drug Administration., 2011). The essential amino acids that compose the protein fraction of edible insects also satisfy the amino acid reference patterns suggested by FAO and WHO for adult men, women, and children (FAO/WHO, 2011). In this context, especially poor people concretely benefit from world trade of insects, as an important source of cheap but valuable proteins (Bukkens, 1997).

Besides nutritional and economic aspects, insect protein fraction was found to have also various functional properties such as antihypertensive, antidiabetic and antioxidant, etc., that even more can make the human consumption of insects promising and helpful (de Castro et al., 2018). More than an intrinsic potential, this ability is mostly induced by the formation of bioactive peptides that are released from the food protein fraction by the work of the hydrolytic enzymes or other factors (Li-Chan, 2015). Today the world is facing an increasing incidence of chronic diseases, including cardiovascular diseases, cancer, etc., and nutrition-related pathologies such as diabetes; at the same time, the interest in natural alternatives to drugs to prevent diseases and maintain human health status is growing considerably. The possibility to exploit natural resources such as food proteins to extract bioactive compounds with the specific ability to prevent pathological conditions is such an attractive prospect for the research world, in particular for the pharmaceutical sector. Even more interesting is the perspective to obtain peptides with beneficial potential with direct action on the human body and health, simply exploiting the human gastrointestinal digestion of food proteins as natural hydrolysis mechanism.

The food products that to date have been investigated as a source of bioactive peptides, especially with antioxidant, antihypertensive and antidiabetic potential, are numerous (Hall et al., 2018; Li-Chan, 2015). They include both animal (such as milk, meat, eggs) and vegetable products (for example soy, pulses, oat etc.) (Udenigwe & Aluko, 2012). As a promising novel protein source for humans, also insects began to attract interest as possible precursors of peptides able to contribute to the maintenance of human health. Despite several biologically active substances have been proved to derive from edible

insects, including antimicrobial compounds and chitin, to date still, relatively few studies have been conducted on the ability to obtain from them bioactive peptides. Similarly, limited research has been performed on *in vivo* and *in vitro* effects of insect protein hydrolysates (MLcek et al., 2014; Nongonierma & FitzGerald, 2017). Further investigations and analysis are needed. Additional information on functionality and bioactivity of the insect fractions could be in part the key for a deeper consumer confidence to accept insects as food.

Antihypertensive, antidiabetic and antioxidant are, among those observed, the biological properties of major interest derived from the protein hydrolysate of some species of insects. According to what was reported by Nongonierma and FitzGerald (2017), until recently, one of the most studied insects as a source of biological peptides able to exert these properties was the *Bombyx mori* (Nongonierma & FitzGerald, 2017). To date, research is focusing on the bioactive potential related to other species. For instance, a recent study on *Alphitobius diaperinus* species has underlined the relevant antioxidant and antihypertensive capacity of its hydrolysate (Sousa et al., 2020). *Tenebio molitor* species has also been recently object of a lot of studies. The results indicate that its protein fraction can be considered a good source of bioactive peptides to be used as functional ingredients (Rivero-Pino et al., 2021; Rivero Pino et al., 2020). In particular, this species was found to be an optimal source of antidiabetic peptides. By investigating the *Tenebrio molitor* protein hydrolysate's activity *in vitro*, indeed, it was observed a good ability in regulating the glycaemic index (Rivero-Pino et al., 2021). The possibility to obtain antidiabetic peptides by natural and cost effectiveness protein sources such as insects could be considered a very interesting chance. In recent decades, in fact, economic well-being has led to increased availability of food, principally in developed countries. This resulted in a tendency to over-eating with an increased risk of overweight and obesity for the population. An increment in prevalence of food-related diseases was the final effect of these excesses. Specifically, a dramatic increase in type 2 diabetes mellitus incidence was observed, also due to increased life expectancy and a more sedentary lifestyle (Maruthur, 2013). The management of this pathological condition generates globally very high therapeutic costs. Thus, one of the

challenges of research is to found alternatives in the treatment of type 2 diabetes, e.g., food derived peptides, which may help to prevent this condition. In this perspective insects could at the same time respond to the need for alternative protein and bioactive peptides. The main quality which makes certain peptides suitable for antidiabetic role is the natural ability to inhibit the dipeptidyl-peptidase IV (DPP-IV), an important enzyme involved in the glycaemic response. It counteracts the activity of GLP-1, a hormone that enables the absorption of glucose by the body (Lacroix et al., 2019). To date, the ability of a wide range of food proteins to naturally inhibit DPP-IV enzyme and, therefore, to regularize the blood glucose levels has been already investigated using both *in vitro* and *in vivo* models (Jao et al., 2015; Lacroix & Li-Chan, 2016). On the contrary, the literature data on the potential of insect protein fraction as a source for antidiabetic peptides are not sufficient. As a future source of alternative food proteins for humans, additional research on the bioactive potential of insect species not yet widely analysed or never investigated (like black soldier fly, as one of the most promising for human nutrition) and on the bioavailability and mechanisms of action of bioactive compounds from insects are needed.

In this regards, **Chapter 5** of the present thesis responds to the need to obtain further information on this opportunity and on the beneficial effects of insect base diet on human health. The ability of protein hydrolysates obtained from Black soldier fly by *in vitro* gastrointestinal digestion to positively modulate the glycaemic response in human GLUTag cell lines, through the DPP-IV activity inhibition and the GLP-1 secretion, was evaluated.

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CHAPTER 2

Effect of the Rearing Substrate on Black Soldier Fly Nutritional Composition with a focus on Total Protein and Amino Acids

The content of this chapter is based on the following publications:

- Barbi, S., Macavei, L. I., Fuso, A., Luparelli, A. V., Caligiani, A., Ferrari, A. M., Maistrello, L., & Montorsi, M. (2020). Valorization of seasonal agri-food leftovers through insects. *Science of the Total Environment*, *709*, 136209. https://doi.org/10.1016/j.scitotenv.2019.136209
- Fuso, A., Barbi, S., Macavei, L. I., Luparelli, A. V., Maistrello, L., Montorsi, M., Sforza, S., & Caligiani, A. (2021). Effect of the rearing substrate on total protein and amino acid composition in black soldier fly. *Foods*, *10*(8), 1773. https://doi.org/10.3390/foods10081773

Abstract

Insects are becoming increasingly relevant as protein sources in food and feed. The Black Soldier Fly (BSF) is one of the most utilized, thanks to its ability to live on many leftovers. Vegetable processing industries produce huge amounts of by-products, and it is important to efficiently rear BSF on different substrates to assure an economical advantage in bioconversion and to overcome the seasonality of some leftovers. This work evaluated how different substrates affect the protein and amino acid content of BSF. BSF prepupae reared on different substrates showed total protein content varying between 35% and 49% on dry matter. Significant lower protein contents were detected in BSF grown on fruit by-products, while higher contents were observed when autumnal leftovers were employed. BSF protein content was mainly correlated to fibre and protein content in the diet. Among amino acids, lysine, valine and leucine were most affected by the diet. Essential amino acids satisfied the Food and Agricultural Organization (FAO) requirements for human nutrition, except for lysine in few cases. BSF could be a flexible tool to bio-convert a wide range of vegetable by-products of different seasonality in a high-quality protein-rich biomass, even if significant differences in the protein fraction were observed according to the rearing substrate.

2.1 Introduction

By 2050, the world will host more than 9 billion people, and the availability of proteins is the main concern in feeding the increasing population, as already pointed out as far back as 1975 by Meyer-Rochow (Meyer-Rochow, 1975). Presently, in Western diets the proteins are predominantly introduced through products of animal origin, although the zootechnical production constitutes an important issue from an ecological point of view due to its impact on the environment (van Huis et al., 2013). A valuable and more sustainable source of protein is represented by insects. They have a high nutritional value (Bessa et al., 2020; X. Chen et al., 2008; Rivero-Pino et al., 2021; Rumpold & Schlüter, 2013) and, compared with traditionally farmed animals, insects have a much higher conversion efficiency and require much less water (van Huis et al., 2013), and their rearing involves much less greenhouse gas emissions (Dennis G.A.B. Oonincx et al., 2010). Nowadays, more than one-third of the edible parts of food produced gets lost or wasted; insects could also bear an important role in managing and valorizing food waste, since they can be reared on a large variety of bio-waste substrates (Cappellozza et al., 2019; Spranghers et al., 2017), thus contributing to their mass reduction and preventing unnecessary waste of resources and further emissions of greenhouse gas (Gustavsson et al., 2011). Therefore, insects fit perfectly in the perspective to valorize bio-waste to create a sustainable food and feed production system that embraces the concept of circular economy and increased sustainability (Bortolini et al., 2020; Madau et al., 2020). The ability of insects to convert waste materials into high-quality nutrients has long been known (Newton et al., 1977), and the Black Soldier Fly (BSF, *Hermetia illucens* L., Diptera, Stratiomiyidae) is known as one of the most efficient bioconverters among insects, being able to reduce the weight of organic waste up to 75% and converting it into a biomass rich in proteins and lipids (Gold et al., 2018). This makes the BSF suitable to be used as feed for farmed animals (Barragan-Fonseca et al., 2017), for biodiesel production (Li et al., 2015) and also for cosmetics or pharmaceuticals industries, thanks to its high chitin content (Gortari & Hours, 2013). All these applications could be simultaneously tackled through an appropriate fractionation method (Caligiani et al., 2018). Last but not least, the residual larval frass can be employed as a quality soil improver

(Choi, 2009; Setti et al., 2019). Many studies have shown that BSF larvae can live on many substrates with different characteristics, ranging from mushrooms (Cai et al., 2019) and winery by-products (Meneguz et al., 2018), restaurant waste (Spranghers et al., 2017), municipal waste (Diener et al., 2011), animal manure (Bortolini et al., 2020; D. G.A.B. Oonincx et al., 2015) and human faeces (Banks et al., 2014). However, according to European legislation (Regulation (EC) No. 1069/2009), when invertebrates are industrially reared, they are considered as "farmed animals"; therefore, the use of animal manure, catering waste or former foodstuffs containing meat and fish as feeding substrates is totally forbidden (EFSA, 2015; European Commission, 2017). As a consequence, despite the lower content of nutrients, vegetable and fruit leftovers are increasingly used as rearing substrates for BSF, also due to their high availability in industrialized regions that are fully compliant with the legislation on feed for farmed animals (Gustavsson et al., 2011). European Food Safety Authority (EFSA) highlighted that no additional microbiological risks are expected for insect rearing on authorized substrates with respect to other animal farming (EFSA, 2015). In addition, a few studies demonstrated that the eventual pesticides and mycotoxins do not accumulate in BSF biomass after the breeding process (Bosch et al., 2017; Lalander et al., 2016; Leni, Cirlini, et al., 2019; Purschke et al., 2017). An important point in the use of vegetal substrates as feed for insects is their seasonal availability. As a matter of fact, most of the vegetal leftovers are not constantly available throughout the year, thus it is important, both from the economic and technical point of view, to understand the feasibility of rearing BSF on a variety of substrates and a combination thereof, in order to have a constant BSF production and composition throughout the year. Many authors have studied the influence of rearing substrates on BSF nutritional composition with a focus on protein fractions. The protein amount showed marked differences, varying from 32 to 58% on dry matter (Gold et al., 2018), while for the amino acid composition, studies in the literature are often inconsistent, and this is true for other insect species as well (Gere et al., 2019). According to Newton et al. (1977) BSF larvae reared on animal manure were lacking some essential amino acids, such as cysteine, methionine, and threonine (Newton et al., 1977), whereas according to Liland et al. (2017)

BSF larvae had sufficient threonine amounts when reared on a conventional diet supplemented with increasing quantities of seaweed (Liland et al., 2017). When fed on a vegetable mix, BSF showed high amounts of aspartic acid, glutamate, and arginine (Cappellozza et al., 2019), but also of leucine (Spranghers et al., 2017). In this paper, a wide range of data was collected on the content and quality of BSF prepupae nutrients, especially protein in relation to the rearing substrates used. A total of 49 different combinations of vegetable rearing substrates were tested in order to obtain the most complete picture of the effect of diet on BSF proteins and on the possibility of using diverse substrates for their rearing. All these vegetable by-products have been chosen focusing on their availability in a specific region (Regione Emilia-Romagna, Italy) and mostly on their seasonal availability. The diets were precisely formulated according to a Design of Experiment approach (Mixture Design) (DoE) (Liland et al., 2017) based on the nutrient composition of each substrate and their seasonal availability, as reported in Barbi et al. (2020) in the optic of a possible scale-up application of BSF rearing on vegetable by-products across the whole year (Barbi et al., 2020). Moreover, the experimental diets allowed us to construct statistically reliable models describing the correlation among food leftovers' composition and amino acid content, utilizing ANOVA to verify if the effects of the main factors and their interaction terms are statistically reliable.

To put this approach into practice and fulfil the requirements of a circular economy, it is of primary importance to verify the composition of BSF reared on the different substrates, and in particular their protein fraction that nowadays is considered as the most valuable component of insects.

2.2 Materials and methods

2.2.1 Materials

Chemicals: AccQ-Fluor reagent kit was obtained from Waters (Milford, MA, USA). DL-norleucine, amino acid standard mixture, L-tryptophan, 5-Methyl-DL-tryptophan, L-Cysteic acid, DL-Dithiothreitol, Ethylenediaminetetraacetic acid (EDTA) and Tris-HCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate was purchased from Biorad (Hercules, CA, USA). All the other solvents, salts, acids and bases were of analytical grade and were purchased from Sigma-Aldrich or Carlo Erba (Milan, Italy).

2.2.2 Leftovers selection and characterization

Agri-food leftovers selection was performed by employing three criteria in order of importance: (i) physical consistency to fit the substrate's needs for insect rearing (e.g. stones have been discarded); (ii) general availability over the year: leftovers that are produced at least in quantities over 5000 ton/year have been considered, in an industrial scale-up perspective; (iii) seasonal availability, to identify at least three diets to be employed at specific times of the year, following the natural availability of agri-food leftovers. In particular, according to the seasonal availability, three possible mixtures were considered: All-year, Summer, Autumn. The organic material was collected from different suppliers of the Emilia Romagna Region (Italy): Agribologna (Bologna, Italy), Conserve Italia (Bologna, Italy), Cooperativa Agricola Brisighellese (Ravenna, Italy). Proximate composition of each selected substrate (Table 2.1) was determined using standard procedures (AOAC Official Method, 2012). Moisture was determined in oven at 105 °C for 24 h. Crude fat content was determined using an automatized Soxhlet extractor (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy) using diethyl ether. Total ash was determined after mineralization at 550 °C in two steps, each one lasting 5 h. Total nitrogen was determined with a Kjeldahl system (DKL heating digester and UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA) using 5.71 as mean nitrogen coefficient conversion. Total fibers were determined by the official method AOAC 991.43 and total polyphenols content by Folin-

Ciocalteu method. Digestible carbohydrates were determined by difference, following the equation:

$$Carbohydrates\ (\%wt)\ =\ 100 - \frac{(Wpr\ +\ Wfa\ +\ Ww\ +\ Wa\ +\ Wpo\ +\ Wfi)}{100}$$

where at the numerator the addition of the weights in grams of protein, fat, water, ash, polyphenols and fiber were considered.

Table 2.1 Leftovers nutrient compositions, grouped by their seasonal availability.

Seasonal mixture	Leftover	Humidity (%)	Fat (%)	N-fraction (protein) (%)	Ash (%)	Fiber (%)	Polyphenol (%)	Carbohydrate (%)
All-year	Exotic Fruit	81.23	0.08	0.72	0.48	2.04	0.012	15.44
	Pineapple	86.51	0.21	0.52	0.62	0.75	0.0018	11.39
	Kiwi	87.54	0.05	0.94	1.73	2.39	0.0042	7.36
	Apple	90.83	0.04	0.35	0.32	1.54	0.0026	6.92
	Melon	93.78	0.08	0.48	0.71	5.61	0.0002	0
Summer	Tomato	79.68	0.37	3.02	0.82	17.23	0.0248	0
	Peach	89.89	0.82	0.87	0.52	3.58	0.0115	4.30
Autumn	Pomace	51.72	3.67	1.97	1.08	33.32	0.0172	8.23
	Legume	60.58	0.7	16.98	1.05	22.63	0.01205	6.33
	Corn	52.2	1.44	11.49	0.97	33.59	0.0123	0.3

2.2.3 BSF rearing substrates design

Selected leftovers (Table 2.1) were combined taking into account their nutritional composition by using a Mixture Design approach, with the aim to enhance the efficiency of BSF rearing in terms of number and weight of obtained prepupae. Mixture Design is a very specific tool in the design of experiments, tailored to optimize proportions among blend ingredients, and therefore widely employed for chemical and pharmaceutical investigations. In this statistical approach two main constraints were considered: the first regarding the relative proportion of the constituents in the mixture, and the second taking

in account the additional boundary constraints on the overall mixture. This approach overcomes the strong simplifications intrinsic to the so-called One Factor-at-A-Time (OFAT) outlook, reducing the number of experimental tests needed to provide the maximum information content on complex issues, subsequently saving both time and resources (Montgomery, 2012). Therefore, to manage this specific experimental region, the Computer Aided D-optimal Design was used (Cornell, 2000). Mixture Model Forms (MMF), most commonly used in fitting data, are the canonical polynomials known as the Scheffé canonical given by (Akalin et al., 2010):

$$MMF = \sum_{i=1}^{q} \beta i \cdot Xi + \sum_{i<j}^{q} Xi \cdot Xj \cdot \beta ij$$

where βi and βij are regression coefficients calculated from the experimental data by multiple regression, and xi and xj are the levels of the independent variables (Akalin et al., 2010). The trial was conducted with three replicates for each diet, allowing the estimation of the model's lack of fit as well as its reliability. The experiments were performed according to the randomized run order to avoid environmental conditioning. To partially overcome the intrinsic limitations of this method (e.g. limitation to combinations at the boundary of the region of interest) an augmented simple centroid model has been selected. Twenty-one experiments, including center points, have been planned and replicated three times for the All-Year mixture, thirteen for the Summer mixture and fifteen for the Autumn mixture.

2.2.4 Insect rearing conditions

Insects belonged to a colony reared in the laboratory of Applied Entomology of the University of Modena and Reggio Emilia (Italy) since 2016, established starting from larvae purchased from CIMI srl (Cuneo, Italy). The colony was maintained in a climatic chamber under controlled conditions (27 ± 0.5 °C, 60–70% RH, 16L:8D). The larvae rearing experiments were conducted under the same conditions in glass containers (20 × 12 × 8 cm). Various quantities of water were added to the substrates to achieve a moisture

content of 70–80%. Selected leftovers were mixed thoroughly using an ordinary ladle to attain certain homogeneity and then added from 250 to 375 g/container to the designated containers following the diets as shown in Table 2.2. One hundred BSF larvae (of second and third stage) were inoculated in each container. A control diet (CNT), the "Gainesville House Fly" diet (50% wheat bran, 30% alfalfa meal and 20% corn meal) was also considered (Hogsette, 1992; Sheppard et al., 2002). The containers were checked twice a week, for a maximum period of 65 days. At each control, the prepupae observed were collected and kept in the freezer (−20 °C) before chemical analysis.

2.2.5 Prepupae proximate composition

Moisture, protein, lipid and ashes were determined using standard procedures as reported in Section 2.1 for vegetable by-products. In particular for the protein fraction, when calculated as BSF total nitrogen content a nitrogen coefficient conversion of 5.71 was used (Caligiani et al., 2018). To consider just the contribution of protein nitrogen in presence of chitin a more specific nitrogen coefficient conversion of 4.76 was instead applied (Janssen et al., 2017).

2.2.6 Amino Acid Profile of BSF Prepupae

2.2.6.1 Sample Preparation

The total amino acid profile was evaluated according to the protocol proposed by Caligiani et al. with some modifications (Caligiani et al., 2018). An amount of 0.5 g of BSF prepupae was hydrolysed with 6 mL of HCl 6 N at 110 ◦C for 23 h, then the internal standard (7.5 mL of 5 mM Norleucine in HCl 0.1 M) was added. Cysteine was determined as cysteic acid after performic acid oxidation followed by acid hydrolysis. In this case, an amount of 0.5 g of BSF was added to performic acid freshly prepared (by mixing 9 volumes of formic acid with 1 volume of hydrogen peroxide) and samples were kept in an ice bath for 16 h at 0 ◦C. Then, 0.3 mL of hydrobromidric acid was added and the bromine formed was removed under nitrogen flow. Then, acid hydrolysis was performed as described above.

2.2.6.2 UPLC/ESI-MS Analysis

The hydrolysed samples were analysed by ultra-performance liquid chromatography with electrospray ionization and mass spectrometry detector (UPLC/ESI-MS, WATERS ACQUITY) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). In particular, UPLC/ESI-MS analysis was performed by using an ACQUITYUPLC separation system with an Acquity BEH C18 column (1.7 µm, 2.1 × 150 mm). The mobile phase was composed of H_2O + 0.2% CH3CN +0.1% HCOOH (eluent A) and CH3CN + 0.1% HCOOH (eluent B). Gradient elution was performed: isocratic 100% A for 7 min, from 100% A to 75.6% A and 24.4% B by linear gradient from 8 to 28 min, isocratic 100% B from 29 to 32 min, isocratic 100% A from 33 to 45 min. The flow rate was set at 0.25 mL/min, injection volume 2 µL, column temperature 35 ◦C and sample temperature 18 ◦C. Detection was performed by using Waters SQ mass spectrometer: the ESI source was in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 150 ◦C, desolvation temperature 300 ◦C, cone gasflow (N_2) 100 L/h, desolvation gas flow (N_2) 650 L/h, full scan acquisition(270–518 m/z) and scan duration 1 s. Calibration was performed with standard solutions prepared mixing norleucine, amino acids hydrolysate standard mixture, cysteic acid and deionized water.

2.2.6.3 Tryptophan Determination

Total tryptophan was determined following the protocol proposed by DelgadoAndrade et al. with some modifications (Delgado-Andrade et al., 2006). An amount of 0.2 g of sample was weighed and dissolved in 3 mL of 4N NaOH. An amount of 150 µL of 5-methyl-tryptophan (16 mg/100 mL), used as internal standard, was added and mixed. Hydrolysis was then carried out for 18 h at 110 ◦C. The hydrolysates were aerated and cooled, then carefully acidified to a pH 6.5 with HCl 37%, diluted to 25 mL with sodium borate buffer (0.1 M, pH 9.0) and allowed to stand for 15 min. Samples were finally centrifuged at 4000 rpm for 5 min and supernatant filtered through a 0.45 µm nylon filter membrane into UPLC vials. UPLC/ESI-MS analysis was performed as for the other amino acids.

2.2.7 Statistical Analysis

All analyses on prepupae were carried out in duplicate. Data are expressed as the mean ± standard deviation. Protein and amino acid data were subjected to one-way analysis of variance (ANOVA) followed by a Tukey post hoc test using IBM SPSS software version 21.0 (SPSS Inc., Chicago, IL, USA) to determine differences between samples. Significant differences were compared at a level of $p < 0.05$. The Pearson correlation coefficient was calculated to evaluate the linear correlations between the nutrients in rearing substrates and protein amounts in BSF prepupae biomass. Both values were taken in absolute terms, calculated according to the following equations:

$$BSF\ total\ protein\ content = \frac{Protein\% * total\ grams\ of prepupae\ at\ the\ end\ of\ the\ breeding}{100}$$

$$Leftovers\ total\ nutrient\ content\ = \frac{nutrient\%\ in\ diet * total\ grams\ of\ diet}{100}$$

The second equation was applied separately for each nutrient present in the diet: proteins, lipids, fibers, carbohydrates, ashes and polyphenols. Principal Component Analysis (PCA) was carried out on amino acid data using IBM SPSS software version 21.0 (SPSS Inc., Chicago, IL, USA). PCA was performed through a 52-point matrix (49 samples plus 3 replicates of the control substrate, one for each seasonal period) and 19 variables (18 amino acids and total protein content), and the principal components were derived with the correlation method. As an unsupervised learning approach, PCA allows us to describe the variation in the dataset. Data were visualised by plotting the score plot and the loading plot, the latter allowing the identification of the amino acids having influences on specific grouping of samples according to the rearing substrate. The Design Expert 12.0 (Stat-Ease) code was used both to set up the experimental plan and to analyse the results. A mixture design was selected to obtain predictive reliability on the effect of the leftovers' composition on the amino acid content of BSF's prepupae. Six factors were considered: proteins, fibers, carbohydrates, polyphenols, lipids, and ashes, from which the

experimental plan of Table 2.2 was derived. ANOVA was employed to estimate the influence of each factor over the responses observing the p-values (α-level of 0.05) and F-tests. The quality of the fit in terms of regression analysis and the predictive power of the model were evaluated by using the R2 and Pred-R2, respectively. R2 is the proportion of the dependent variable's variance predictable from the independent variables. In a similar way, Pred-R2 shows how well the model can predict the responses for new observations. Response contour plots were used as functional tools in explaining graphically the role of the main components on the final considered properties (Montgomery, 2012).

Table 2.2: Formulation and proximate composition of the rearing substrates, and total weight of corresponding prepupae biomass

	REARING SUBSTRATES FORMULATION											PROXIMATE COMPOSITION OF REARING SUBSTRATES FORMULATED							TOTAL PREPUPAE BIOMASS (G)
	Entomology code	Exotic fruits (%)	Pineapple (%)	Kiwi (%)	Apple (%)	Melon (%)	Peach (%)	Tomato (%)	Legume (%)	Corn (%)	Pomace (%)	Humidity (%)	Lipid (%)	Crude protein (%)	Ash (%)	Polyphenols (%)	Dietary fibre (%)	Non-structural carbohydrate (%)	
All-Year group	A	50	0	0	0	50	/	/	/	/	/	87,51	0,08	0,60	0,60	0,01	3,83	7,72	26,25
	B	10	10	60	10	10	/	/	/	/	/	87,76	0,07	0,77	1,25	0,00	2,43	7,79	19,47
	C	0	100	0	0	0	/	/	/	/	/	86,51	0,21	0,52	0,62	0,00	0,75	11,39	17,15
	D	0	0	0	0	100	/	/	/	/	/	93,78	0,08	0,48	0,71	0,00	5,61	0,00	22,54
	E	0	0	0	100	0	/	/	/	/	/	90,83	0,04	0,35	0,32	0,00	1,54	6,92	20,4
	F	100	0	0	0	0	/	/	/	/	/	81,23	0,08	0,72	0,48	0,01	2,04	15,44	18,31
	G	20	20	20	20	20	/	/	/	/	/	87,98	0,09	0,60	0,77	0,00	2,47	8,22	23,51
	H	50	0	50	0	0	/	/	/	/	/	84,39	0,07	0,83	1,11	0,01	2,22	11,40	20,83

J	0	50	0	0	50	/	/	/	/	/	90,15	0,15	0,50	0,67	0,00	3,18	5,70	23,44
L	0	0	50	0	50	/	/	/	/	/	90,66	0,07	0,71	1,22	0,00	4,00	3,68	20,93
N	0	50	0	50	0	/	/	/	/	/	88,67	0,13	0,44	0,47	0,00	1,15	9,16	22,32
P	10	10	10	60	10	/	/	/	/	/	89,40	0,07	0,48	0,55	0,00	2,00	7,57	22,77
Q	0	0	0	50	50	/	/	/	/	/	92,31	0,06	0,42	0,52	0,00	3,58	3,46	24,57
R	10	10	10	10	60	/	/	/	/	/	90,88	0,09	0,54	0,74	0,00	4,04	4,11	25,34
S	0	50	50	0	0	/	/	/	/	/	87,03	0,13	0,73	1,18	0,00	1,57	9,38	19,66
T	50	50	0	0	0	/	/	/	/	/	83,87	0,15	0,62	0,55	0,01	1,40	13,42	20,69
U	0	0	100	0	0	/	/	/	/	/	87,54	0,05	0,94	1,73	0,00	2,39	7,36	13,06
V	50	0	0	50	0	/	/	/	/	/	86,03	0,06	0,54	0,40	0,01	1,79	11,18	22,07
X	60	10	10	10	10	/	/	/	/	/	84,60	0,09	0,66	0,63	0,01	2,25	11,83	22,59
Y	10	60	10	10	10	/	/	/	/	/	87,24	0,15	0,56	0,70	0,00	1,61	9,81	20,6
Z	0	0	50	50	0	/	/	/	/	/	89,19	0,05	0,65	1,03	0,00	1,97	7,14	19,59

	Entomology code	Exotic fruits (%)	Pineapple (%)	Kiwi (%)	Apple (%)	Melon (%)	Peach (%)	Tomato (%)	Legume (%)	Corn (%)	Pomace (%)	Humidity (%)	Lipid (%)	Crude protein (%)	Ash (%)	Polyphenols (%)	Dietary fibre (%)	Non-structural carbohydrate (%)	
Summer group	A	0	/	/	/	0	67	33	/	/	/	86,48	0,67	1,59	0,62	0,02	8,13	2,87	39,54
	B	0	/	/	/	0	100	0	/	/	/	89,89	0,82	0,87	0,52	0,01	3,58	4,30	36,69
	C	20	/	/	/	47	16	17	/	/	/	88,27	0,25	1,01	0,65	0,01	6,49	3,81	36,9
	D	10	/	/	/	24	33	33	/	/	/	86,53	0,43	1,48	0,66	0,01	8,45	2,98	39,45
	E	10	/	/	/	23	0	67	/	/	/	83,12	0,28	2,20	0,76	0,02	13,00	1,54	42,42
	F	20	/	/	/	47	33	0	/	/	/	89,97	0,33	0,66	0,60	0,01	4,22	4,52	36,33
	G	5	/	/	/	12	67	16	/	/	/	88,21	0,62	1,18	0,59	0,01	6,01	3,64	37,21
	H	20	/	/	/	47	0	33	/	/	/	86,57	0,18	1,37	0,70	0,01	8,77	3,09	36,37
	I	30	/	/	/	70	0	0	/	/	/	90,02	0,08	0,55	0,64	0,00	4,54	4,63	35,01
	J	0	/	/	/	0	33	67	/	/	/	83,08	0,52	2,31	0,72	0,02	12,68	1,43	41,89
	K	5	/	/	/	11	17	67	/	/	/	83,10	0,40	2,25	0,74	0,02	12,84	1,49	41,01
	L	0	/	/	/	0	0	100	/	/	/	79,68	0,37	3,02	0,82	0,02	17,23	0,00	50,46
	M	10	/	/	/	23	67	0	/	/	/	89,93	0,58	0,76	0,56	0,01	3,90	4,41	39,52

	Entomology code	Exotic fruits (%)	Pineapple (%)	Kiwi (%)	Apple (%)	Melon (%)	Peach (%)	Tomato (%)	Legume (%)	Corn (%)	Pomace (%)	Humidity (%)	Lipid (%)	Crude protein (%)	Ash (%)	Polyphenols (%)	Dietary fibre (%)	Non-structural carbohydrate (%)	
Autumn group	A	4	/	/	/	8,5	/	/	12,5	62,5	12,5	60,37	1,23	8,51	1,06	0,01	26,47	2,40	62,88
	B	15	/	/	/	35	/	/	0	50	0	71,11	0,76	6,02	0,80	0,01	19,06	2,47	67,4
	C	0	/	/	/	0	/	/	0	100	0	52,20	1,44	11,49	0,97	0,01	33,59	0,30	51,47
	D	4	/	/	/	8,5	/	/	12,5	12,5	62,5	69,94	1,43	3,49	1,57	0,01	18,01	5,60	52,14
	E	4	/	/	/	8,5	/	/	62,5	12,5	12,5	64,59	0,86	7,09	1,11	0,01	20,99	5,41	60,95
	F	0	/	/	/	0	/	/	100	0	0	60,63	0,70	8,65	1,05	0,01	22,63	6,33	48,08
	G	0	/	/	/	0	/	/	50	0	50	65,98	1,28	5,05	1,52	0,01	19,64	6,52	55,75
	H	30	/	/	/	70	/	/	0	0	0	90,02	0,08	0,55	0,64	0,00	4,54	4,63	40,60
	I	0	/	/	/	0	/	/	50	50	0	56,42	1,07	10,07	1,01	0,01	28,11	3,32	54,33
	J	15	/	/	/	35	/	/	0	0	50	80,67	0,96	1,00	1,31	0,01	10,60	5,67	40,64
	K	19	/	/	/	43,5	/	/	12,5	12,5	12,5	79,28	0,55	3,04	0,90	0,01	11,95	4,56	55,55
	L	0	/	/	/	0	/	/	0	0	100	71,33	1,85	1,45	1,98	0,02	16,66	6,71	18,46

M	0	/	/	/	0	/	/	0	50	50	61,76	1,64	6,47	1,47	0,01	25,13	3,51	58,68
N	15	/	/	/	35	/	/	50	0	0	75,32	0,39	4,60	0,85	0,01	13,58	5,48	56,91
O	7,5	/	/	/	17,5	/	/	25	25	25	68,54	1,02	5,53	1,16	0,01	19,35	4,49	65,64

2.3 Results and discussion

2.3.1 Rearing performance and prepupae gross composition analysis

The prospect of a massive BSF prepupae exploitation to enhance organic wastes leads to the need to have leftovers to be used as substrates for SBF prepupae growth always available, no matter the period of the year. Thus, according to seasonal availability, organic leftovers from Agri-food industries were grouped to form three specific seasonal diets (All-year, Summer and Autumn).

In addition, for each diet different proportions of its ingredients were tested to be able to optimize insect growth. The different leftover combinations were formulated based on their nutritional composition, by using a Mixture design approach (as explained in paragraph 2.2.3 of materials and methods), aimed to maximize the BSF breeding performances.

In Tables 2.3, 2.4 and 2.5 all the performed experiments related to the optimized ingredients proportions and the respective obtained results have been summarized in terms of rearing performance and nutritional composition of the prepupae obtained.

Table 2.3 Complete experimental plan generated by combining all year available leftovers.

						Prepupae rearing parameters and nutritional composition						
RUN	A:Exotic Fruit	B:Pineapple	C:Kiwi	D:Apple	E:Melon	P Weight (g)	P Number	Days	Humidity (%)	Fat (%)	N-fraction (protein + chitin) (%)	Ash (%)
1	50	0	0	0	50	0.0895	95.0	29.0	71.14	6.52	14.04	2.60
2	10	10	60	10	10	0.0665	83.3	29.0	70.95	7.70	13.49	4.80
3	0	100	0	0	0	0.0783	60.3	33.7	72.27	5.01	13.39	4.86
4	0	0	0	0	100	0.0741	99.3	24.0	70.78	5.05	15.06	3.50
5	0	0	0	100	0	0.0665	89.3	25.3	70.61	7.01	13.92	3.69
6	100	0	0	0	0	0.0601	59.0	31.3	69.66	9.60	13.81	3.75
7	20	20	20	20	20	0.0766	93.7	26.7	69.79	8.84	12.91	3.78
8	50	0	50	0	0	0.0643	64.7	25.3	69.94	8.42	12.87	4.74
9	0	50	0	0	50	0.0839	89.7	29.0	71.51	5.99	13.93	3.80
10	0	0	50	0	50	0.0705	81.3	26.7	70.01	6.00	13.45	4.48
11	0	50	0	50	0	0.0750	91.3	28.0	73.77	7.70	13.87	3.82
12	10	10	10	60	10	0.0745	87.7	28.7	69.25	7.27	14.19	4.00
13	0	0	0	50	50	0.0857	94.3	26.3	70.24	7.52	14.27	4.10
14	10	10	10	10	60	0.0882	94.0	25.3	69.69	5.83	13.92	3.24
15	0	50	50	0	0	0.0753	67.0	27.7	68.75	7.55	14.51	5.11
16	50	50	0	0	0	0.0668	83.7	25.3	72.40	8.81	14.13	3.77
17	0	0	100	0	0	0.0518	56.7	25.3	71.28	7.85	13.13	5.77
18	50	0	0	50	0	0.0689	79.7	29.0	68.84	9.52	13.94	4.70

19	60	10	10	10	10	0.0651	87.0	32.3	70.38	8.52	13.55	3.93
20	10	60	10	10	10	0.0787	80.3	26.7	71.40	7.48	14.16	3.90
21	0	0	50	50	0	0.0691	80.0	28.0	72.68	7.03	13.07	4.12
CNT	/	/	/	/	/	0.1654	99.7	18.0	68.73	12.00	14.95	2.33

Table 2.4 Complete experimental plan generated by combining Summer available leftovers.

				Prepupae rearing parameters and nutritional composition							
RUN	A:All-Year Mix	B:Peach	C:Tomato	P Weight (g)	P Number	Days	Nwater (mL)	Humidity (%)	Fat (%)	N-fraction (%)	Ash (%)
1	0	67	33	0.1416	93.0	26.0	0.00	72.83	13.67	15.65	2.07
2	0	100	0	0.1399	87.3	33.0	0.20	72.03	13.63	15.52	1.68
3	67	17	17	0.1324	93.0	20.3	0.50	71.61	12.08	15.00	2.87
4	33	33	33	0.1324	99.3	21.3	0.58	72.23	12.05	15.26	3.27
5	33	0	67	0.146	97.0	18.0	0.75	69.41	10.63	15.64	3.02
6	67	33	0	0.1293	93.7	23.7	0.42	73.01	10.67	14.57	3.06
7	17	67	17	0.1396	89.0	25.0	0.50	73.07	14.16	16.36	2.40
8	67	0	33	0.1284	94.7	18.0	0.42	70.66	7.46	15.26	3.26
9	100	0	0	0.1275	91.7	18.0	0.75	69.99	8.96	14.79	3.33
10	0	33	67	0.1455	96.0	18.0	0.50	71.74	12.17	14.82	2.68
11	17	17	67	0.1438	95.0	18.0	0.50	71.80	12.61	15.85	2.66
12	0	0	100	0.1699	99.0	18.0	1.00	71.34	11.36	15.66	2.94
13	33	67	0	0.1397	94.3	33.0	0.17	73.47	12.51	15.96	1.99
CNT	/	/	/	0.1951	98.6	12.0	1.00	70.77	11.67	16.33	3.21

Table 2.5. Complete experimental plan generated by combining the autumn available leftovers.

					Prepupae rearing parameters and nutritional composition								
RUN	A:Legume	B:Corn	C:Pomace	D: All-Year Mix	P Weight (g)	P Number	Days	Nsubstrate (g)	Nwater (mL)	Humidity (%)	Fat (%)	N-fraction (%)	Ash (%)
1	13	63	13	13	0.2123	98.7	14.0	18.06	0.41	71.33	11.97	16.91	1.08
2	0	50	0	50	0.2268	99.0	13.0	19.44	0.19	72.83	11.57	16.94	1.18
3	0	100	0	0	0.1831	93.7	13.0	19.44	0.47	70.56	10.18	18.44	0.93
4	13	13	63	13	0.1861	94.7	19.0	19.74	0.19	70.63	8.69	16.50	1.61
5	63	13	13	13	0.2065	98.3	13.0	19.44	0.33	71.29	10.97	16.20	1.55
6	100	0	0	0	0.1775	90.0	14.0	18.06	0.45	70.00	7.04	17.28	1.40
7	50	0	50	0	0.1889	98.3	15.0	16.67	0.26	72.53	6.24	16.47	1.53
8	0	0	0	100	0.1368	99.0	19.0	19.74	0.06	74.75	8.94	15.32	1.84
9	50	50	0	0	0.1929	93.7	12.0	20.83	0.47	71.56	7.43	17.63	1.27
10	0	0	50	50	0.1448	93.3	26.0	14.42	0.39	69.97	4.94	14.76	2.37
11	13	13	13	63	0.1865	99.3	14.0	18.06	0.14	69.27	7.59	17.77	2.31
12	0	0	100	0	0.1111	62.3	47.0	7.98	1.24	70.81	5.63	14.76	3.82
13	0	50	50	0	0.2036	96.0	15.0	16.67	0.45	71.14	7.76	17.41	1.31
14	50	0	0	50	0.1915	99.0	13.0	19.44	0.19	74.45	7.69	16.94	1.05
15	25	25	25	25	0.2188	100.0	12.0	20.83	0.21	72.24	8.73	17.73	1.96
CNT	/	/	/	/	0.1667	97.3	20.2	12.37	0.05	71.58	7.86	16.81	2.46

In general, all the substrates tested in this study allowed BSF growth and development to prepupae, which is consistent with the results obtained by several authors using rearing substrates such as kitchen waste and vegetables (Chia et al., 2018; Dennis G.A.B. Oonincx et al., 2015). Then, the composition of rearing substrates, both in terms of leftover combinations and specific nutrient composition (Table 2.2) and the mean composition of the diets according to the seasonality (substrates available all year, substrates available in summer and substrates available in autumn) (Table 2.6) were also evaluated and compared with the BSF standard diet (Gainsville diet, CTR). The three groups of tested diets differed between each other and in respect to the control diet. The differences in diet compositions are related to the vegetable by-products employed: the agri-food components belonging to single diets are summerized in Table 2.1. The different ingredients employed in the three groups are also related to a different proximate composition of the diets, as reported in Table 2.6.

Table 2.6. Mean percentage composition of the different diets, expressed as g/100 g dry matter and corresponding amount of total prepupal biomasses obtained (g) starting from 300 Black Soldier Fly (BSF) larvae. Nd = not detected.

	ALL-YEAR (n=21)	SUMMER (n=13)	AUTUMN (n=15)	CTR
Lipid	0.78 ± 0.35	3.28 ± 2.12	3.18 ± 1.46	3.37 ± 0.1
Ashes	6.66 ± 3.20	4.87 ± 0.71	4.02 ± 1.59	5.9 ± 0.5
Fibers	23.1 ± 17.51	56.86 ± 14.79	56.69 ± 6.53	41 ± 2.5
Polyphenols	0.03 ± 0.02	0.09 ± 0.02	0.04 ± 0.01	Nd
Protein	5.07 ± 1.20	9.97 ± 2.71	16.03 ± 6.57	17.28 ± 1
Available carbohydrates	64.36 ± 20.07	24.93 ± 15.61	17.05 ± 10.72	32.61 ± 1

Data on the prepupae raised on all-year leftovers, as shown in Table 2.3, demonstrate that they are lighter in weight (~58%) and less in number (~17%) when compared with the control group. Conversely, basically no differences can be observed when employing Summer available leftovers (Table 2.4) or the control substrate during rearing, leading in both cases to very similar prepupae weight, prepupae numbers and rearing days. Autumn-available leftovers (Table 2.5) led to further different results, in fact showing an overall decrease in rearing days (~25%) with respect to the control group. From the results a general estimate of the BSF nutrient evaluation can be drawn. As is possible to see from Table 2.6, the All-Year diets are rich in available carbohydrates and poor in protein and lipids, while the Summer and Autumn groups contain larger amounts of fibers and protein, the latter especially represented in the Autumn group. BSF reared on All-year available leftovers (mainly fruit by-products) show in general a significantly lower amount of fat (~5%) with respect to the control group. Regarding N-fraction (comprising protein and chitin), an overall slight increase (~1%) with respect to the control diet is observed when combinations of Autumn-available leftovers (comprising cereal and legume residues) are employed as rearing substrate. Looking more into details of the connection with the above reported performances and substrate composition, further considerations can be drawn. The presence of only fruit by-products in the All-year mixture (Table 2.1) led to lower weight of prepupae as well as longer growth times, as indicated in Table 2.3 and summarized in Table 2.6. This can be due to the fact that these substrates, even if in some cases containing good amounts of carbohydrates, are very poor in proteins and lipids. This result is generally consistent with the fact that carbohydrates alone are not sufficient for insect rearing (Cammack & Tomberlin, 2017). Accordingly, the fat content of BSF reared on this substrate is generally lower than in the control. Considering instead the average N-fraction and Ash content of the prepupae reared on this substrate, similar results to the control group were obtained. Considering the Summer-available leftovers, a general increase of the rearing performance has been reported with respect to the previous one, as showed in Table 2.4 and summarized in Table 2.6. This result is consistent with the fact that tomato leftovers has been included in this group of data, and its nutritional content is

different from the leftovers previously employed (All-year), due to the higher amount of N-fraction (proteins) and fibers (Table 2.1). The presence of fibers in some vegetable substrates, together with proteins, seems to reduce development time and leads to a higher body weight, likely because the BSF gut microbiota includes species able to degrade cellulose (Lee et al., 2014), thus increasing the availability of nutrients (Cammack & Tomberlin, 2017; Kim et al., 2014; Lee et al., 2014; Meneguz et al., 2018). In a similar way, but to a lesser extent, peach as rearing substrate is able to provide a slightly higher amount of fat for BSF development, leading to an overall more desirable prepupae nutritional content, very similar to the one obtained in the control group and more favorable for employment as feed with respect to All-year best mixture. The Autumn available leftovers mixture led to even better results, as a result of a wider nutritional content of the Autumn available leftovers, obtaining values very similar to the control group. Indeed, corn and legume have a strong positive effect on prepupae rearing, likely because of the higher N-fraction (protein) and fiber content compared to the other investigated leftovers. On the contrary, increasing the amount of olive pomace in the Autumn diet resulted in a negative effect on BSF rearing. This substrate, characterized by a high fat content and very low N-fraction, proved to be unbalanced for BSF development, and possibly the high amount and the specific type of fibers and polyphenols of pomace had a detrimental effect on larval growth.

2.3.2 Effect of the rearing substrate composition on BSF prepupae total protein content

Given that the composition of the diets influences the growth and composition of the prepupae, the target of this work was also to verify how individual seasonal diets based on their average nutritional properties, and in turn the different growth performance of prepupae (low, medium and high biomass weights), influence the final BSF protein quality and its total amount. No longer considering nitrogenous compounds in general, as had been done in Table 2.3, 2.4 and 2.5, but wanting to calculate just the contribution of protein nitrogen, the total protein content of each BSF prepupa reared on the different substrates, reported in Figure 2.1, was determined by using as nitrogen coefficient conversion a more specific value for BSF proteins only, corresponding to 4.67 (Janssen et al., 2017). (see Table

2.2 for the details about substrate typologies, composition and sample codes). Protein amount, calculated as g/100 g of BSF on dry matter (% DM), varied in the range of 35% to 49.5%. These values agree with the studies in the literature, reporting a range of 32–58% (Gold et al., 2018).

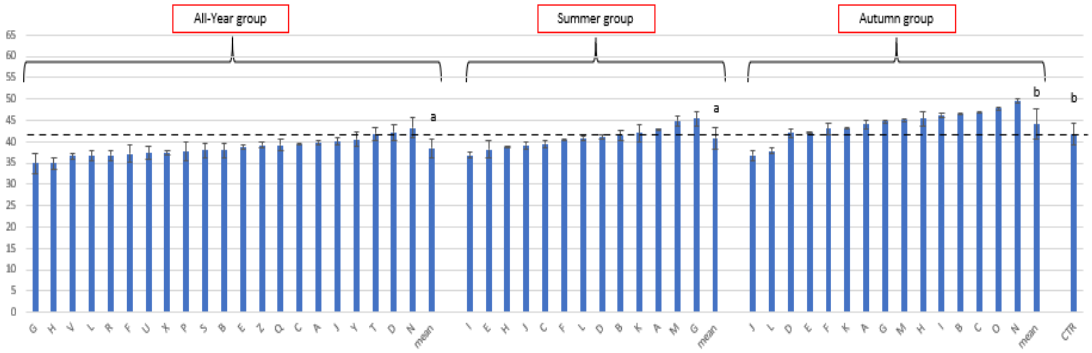

Figure 2.1. Total protein content (Nx4.67, g/100 g DM) for each BSF sample grown on the 49 diets considered and compared with the control samples (dotted line). Each datum is the mean of two replicates. Global mean of each group was compared with one-way ANOVA (p-level 0.05). Different letters (a,b) on the bars indicate significantly different values.

In general, as already observed in the evaluation of the total N fraction done in the paragraph above, most of the samples from the All-Year and Summer groups contained lower amounts of protein compared to the Autumn group samples, that often contained equal or even higher amounts of protein with respect to the control. To better understand which relationship exists between the various diets administered and the production of protein in the prepupae biomass, we correlated the latter and the single nutrients of the diets through linear correlation analysis. A positive, although moderate, correlation was observed between the lipid content of the diet and the protein content of prepupae ($R = 0.54$; $p < 0.001$), while non-structural carbohydrates (digestible carbohydrates) showed a moderate negative one ($R = -0.56$, $p < 0.001$). On the contrary, polyphenols ($R = 0.39$, $p < 0.001$) and ashes ($R = 0.17$, $p = 0.37$) in the diet did not affect the protein content of BSF prepupae. A significant positive correlation was detected between the BSF prepupae protein content and the fibers content of the diet ($R = 0.87$, $p < 0.001$, Figure 2.2a). Fibers are typically difficult to degrade for most insects, but this positive correlation could be the result of the increased availability of nutrients due to the action of microorganisms able to

hydrolyse cellulose, both endogenous and exogenous (i.e., already present in the rearing substrates) (Jeon et al., 2011; Kim et al., 2014; Meneguz et al., 2018; Terra & Ferreira, 2012). Indeed, previous studies have identified cellulase genes in the gut microflora of BSF larvae (Jeon et al., 2011; Lee et al., 2014). Thus, a likely hypothesis for the correlation found might be that the ability of using more and better fibre biomass leads to better larval growth, which in turn also means a higher amount of protein produced. A positive correlation was also identified between the BSF prepupae protein content and the diet's protein content in absolute terms ($R = 0.84$, $p < 0.001$, Figure 2.2b), confirming previous findings (Meneguz et al., 2018; D. G.A.B. Oonincx et al., 2015).

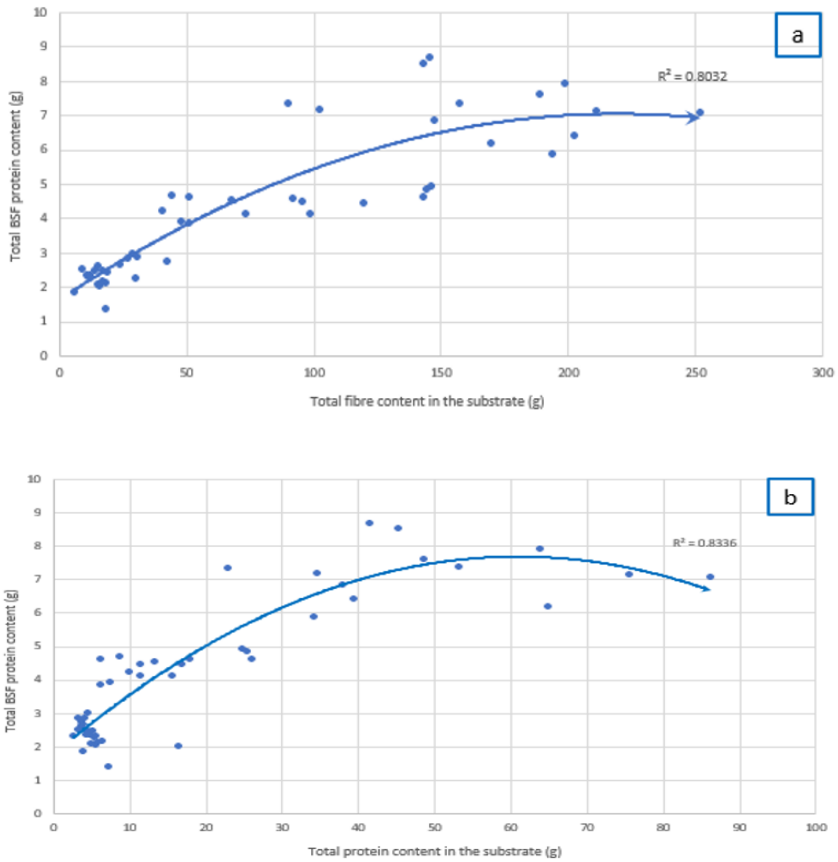

Figure 2.2 Correlation between total protein content (absolute amount) in the BSF prepupae biomass recorded in each experiment (starting from 300 BSF larvae) and (a) total fibre and (b) total protein contained in the rearing substrate (absolute amount).

Observing qualitatively the data in Figure 2.2 a,b, a lack of a linear correlation (a plateau is reached) seems evident, especially when a high amount of fibre or protein was present in the diet, suggesting that there is a specific level of these nutrients in the substrates allowing them to reach the maximum amount of protein in insects. More specifically, our data show that by increasing the amount of vegetable protein in the diet, BSF larvae convert progressively a smaller part of it into their own animal protein. In fact, whilst for low-protein diets they were able to convert more than 90%, for the most protein-rich diets, this percentage dropped to 10% (Figure 2.3). This suggests that for BSF larvae growth and protein content, the amount of protein in the rearing substrate is very important until a minimum threshold is reached, and then it becomes less relevant. These experimental data are in agreement with a recent work about digestive enzyme expression and production in BSF (Bonelli et al., 2020). In this paper, the authors clearly demonstrate that the midgut of H. illucens larvae is able to adapt to diets with different nutrient content; an increase in proteolytic activity together with a decrease in α-amylase and lipase activity was observed as a consequence of nutritionally poor diet. Moreover, Barragán Fonseca (2018) demonstrated that larvae feeding on substrates rich in protein have a higher lipid content, and thereby a reduced protein content (in % dry mass) (Barragán-Fonseca, 2018). Consequently, in order to obtain prepupae with a high protein content, according to Figure 2.3, a good compromise to maintain an advantageous conversion rate would be to rear them on a substrate containing 7% by weight of protein. This value is also in accordance with a previous study (Gere et al., 2019).

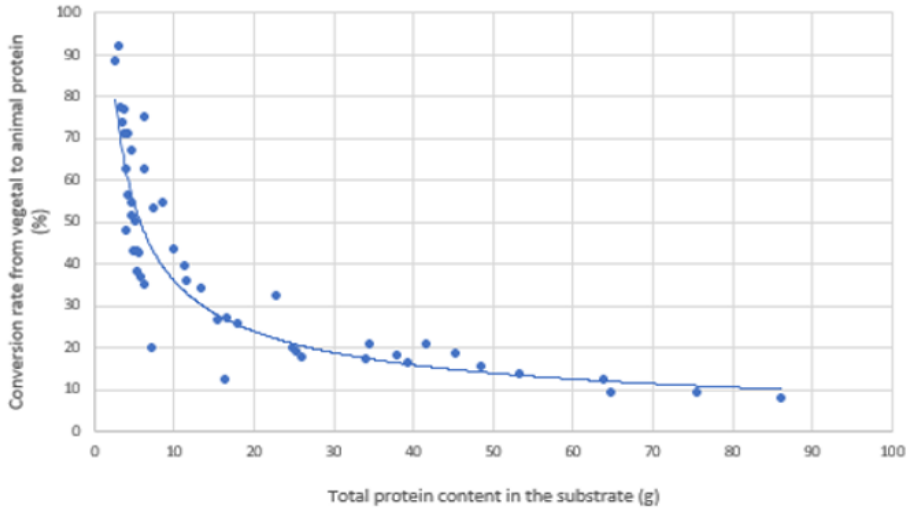

Figure 2.3 Conversion rate (%) of vegetable protein present in the substrate into BSF protein (absolute amounts, starting from 300 BSF larvae.

2.3.3 BSF amino acid content

To better verify the influence of the rearing substrate composition on the BSF protein nutritional value, further insights into the complete amino acid profile were provided. As a matter of fact, information on the nutritional quality of proteins, and therefore on their amino acid composition, is of utmost importance to understand the possible uses of the BSF protein fraction. Results on the complete total amino acid profile of the BSF prepupae are reported in Table 2.7.

Table 2.7: Complete amino acid composition (mg AA/g protein) of BSF prepupae grown on different diets

		mg AA/g protein																	
	Entomology code	**ala**	**asp + asn**	**arg**	**Gly**	**his**	**ile**	**leu**	**met**	**phe**	**pro**	**ser**	**thr**	**val**	**lys**	**tyr**	**glu + gln**	**trp**	**cys**
ALL-YEAR GROUP	A	65 ± 6	113 ± 14	69 ± 8	60 ± 7	23 ± 16	42 ± 2	67 ± 2	19 ± 1	42 ± 7	72 ± 8	53 ± 2	41 ± 0	60 ± 2	60 ± 13	61 ± 14	124 ± 1	14 ± 2	14 ± 0
	B	62 ± 7	114 ± 16	71 ± 18	50 ± 0	33 ± 5	42 ± 1	70 ± 0	19 ± 0	37 ± 5	73 ± 5	48 ± 1	40 ± 2	60 ± 1	59 ± 8	56 ± 7	131 ± 18	18 ± 1	18 ± 0
	C	62 ± 2	92 ± 19	80 ± 7	57 ± 7	37 ± 13	43 ± 2	69 ± 4	19 ± 0	48 ± 12	69 ± 2	50 ± 3	41 ± 3	60 ± 1	59 ± 15	73 ± 16	108 ± 19	16 ± 3	17 ± 2
	D	63 ± 2	106 ± 2	72 ± 10	56 ± 0	30 ± 1	42 ± 1	70 ± 0	22 ± 2	49 ± 0	68 ± 4	49 ± 0	41 ± 1	61 ± 1	57 ± 8	69 ± 11	109 ± 8	17 ± 0	17 ± 1
	E	65 ± 3	118 ± 5	71 ± 12	51 ± 3	36 ± 5	41 ± 2	69 ± 1	17 ± 1	38 ± 1	70 ± 4	47 ± 2	39 ± 4	59 ± 3	61 ± 2	59 ± 3	126 ± 2	15 ± 1	19 ± 2
	F	65 ± 2	111 ± 9	70 ± 5	51 ± 1	35 ± 5	45 ± 1	72 ± 4	18 ± 1	39 ± 2	72 ± 1	49 ± 3	38 ± 0	60 ± 3	64 ± 8	58 ± 0	120 ± 0	14 ± 0	17 ± 2
	G	72 ± 12	107 ± 18	63 ± 2	54 ± 7	27 ± 4	46 ± 5	75 ± 9	17 ± 0	40 ± 1	71 ± 3	46 ± 6	36 ± 8	66 ± 4	70 ± 8	51 ± 10	128 ± 7	14 ± 1	16 ± 0
	H	61 ± 4	105 ± 6	78 ± 1	53 ± 2	33 ± 0	43 ± 2	71 ± 2	18 ± 1	43 ± 1	69 ± 3	50 ± 2	38 ± 1	60 ± 1	63 ± 1	61 ± 4	121 ± 4	15 ± 1	19 ± 3
	J	64 ± 3	103 ± 8	75 ± 3	54 ± 10	37 ± 2	43 ± 2	72 ± 1	18 ± 5	42 ± 6	72 ± 2	56 ± 7	38 ± 0	60 ± 3	59 ± 15	66 ± 13	117 ± 16	7 ± 1	16 ± 2
	L	66 ± 3	115 ± 2	74 ± 4	54 ± 9	34 ± 5	39 ± 3	71 ± 3	16 ± 1	35 ± 3	72 ± 3	47 ± 2	35 ± 2	59 ± 5	64 ± 17	56 ± 9	128 ± 8	15 ± 1	20 ± 1
	N	67 ± 3	110 ± 5	73 ± 2	47 ± 1	31 ± 3	45 ± 4	73 ± 6	16 ± 0	39 ± 5	71 ± 1	47 ± 1	38 ± 1	63 ± 4	72 ± 3	53 ± 1	129 ± 14	12 ± 2	16 ± 4
	P	66 ± 2	109 ± 7	71 ± 7	50 ± 2	33 ± 3	44 ± 1	71 ± 0	20 ± 1	42 ± 3	68 ± 3	50 ± 0	42 ± 2	63 ± 1	60 ± 6	62 ± 1	116 ± 5	17 ± 2	15 ± 3
	Q	63 ± 3	106 ± 3	78 ± 6	50 ± 3	37 ± 5	43 ± 1	73 ± 3	21 ± 1	41 ± 1	64 ± 0	50 ± 2	40 ± 0	60 ± 0	60 ± 4	59 ± 3	118 ± 4	19 ± 1	19 ± 2
	R	64 ± 4	106 ± 3	77 ± 2	50 ± 3	33 ± 5	41 ± 2	72 ± 2	18 ± 0	42 ± 2	68 ± 4	49 ± 3	40 ± 0	58 ± 0	65 ± 2	63 ± 5	119 ± 1	15 ± 1	21 ± 3
	S	64 ± 4	108 ± 1	74 ± 5	51 ± 3	33 ± 0	42 ± 1	71 ± 3	17 ± 2	42 ± 1	68 ± 4	49 ± 3	40 ± 1	59 ± 2	61 ± 10	63 ± 1	119 ± 1	16 ± 0	22 ± 0
	T	65 ± 1	111 ± 6	73 ± 0	51 ± 2	35 ± 1	43 ± 1	71 ± 0	18 ± 1	41 ± 2	64 ± 1	48 ± 2	41 ± 1	62 ± 1	64 ± 8	63 ± 3	116 ± 7	14 ± 0	20 ± 3
	U	62 ± 2	100 ± 5	68 ± 2	50 ± 5	31 ± 3	44 ± 0	73 ± 2	19 ± 2	45 ± 11	72 ± 2	53 ± 3	42 ± 1	61 ± 1	61 ± 17	63 ± 15	125 ± 12	12 ± 0	20 ± 1
	V	61 ± 1	105 ± 9	79 ± 3	48 ± 1	38 ± 1	42 ± 1	71 ± 0	19 ± 0	42 ± 1	67 ± 4	51 ± 4	40 ± 0	57 ± 0	62 ± 3	60 ± 2	122 ± 7	16 ± 2	19 ± 1

	X	60 ± 2	101 ± 15	82 ± 6	51 ± 6	36 ± 2	44 ± 3	72 ± 1	19 ± 1	47 ± 5	67 ± 3	47 ± 2	39 ± 0	61 ± 4	55 ± 8	70 ± 11	110 ± 0	18 ± 4	20 ± 2
	Y	61 ± 6	105 ± 12	80 ± 1	49 ± 2	38 ± 7	44 ± 1	72 ± 1	18 ± 2	42 ± 7	68 ± 4	49 ± 2	40 ± 2	61 ± 1	62 ± 12	66 ± 9	114 ± 9	12 ± 1	17 ± 2
	Z	63 ± 4	104 ± 8	77 ± 6	48 ± 3	33 ± 5	43 ± 3	72 ± 0	18 ± 0	38 ± 5	71 ± 0	50 ± 4	39 ± 5	60 ± 4	69 ± 3	55 ± 7	125 ± 5	14 ± 1	21 ± 2
	Entomology code	**ala**	**asp + asn**	**arg**	**gly**	**his**	**ile**	**leu**	**met**	**phe**	**pro**	**ser**	**thr**	**val**	**lys**	**tyr**	**glu + gln**	**trp**	**cys**
SUMMER GROUP	A	64 ± 3	96 ± 1	76 ± 6	48 ± 3	41 ± 2	44 ± 1	75 ± 1	18 ± 1	42 ± 5	70 ± 0	46 ± 2	36 ± 2	59 ± 2	66 ± 11	62 ± 4	118 ± 3	19 ± 1	20 ± 2
	B	67 ± 3	91 ± 3	77 ± 2	47 ± 1	41 ± 2	46 ± 0	84 ± 8	15 ± 2	38 ± 3	71 ± 4	44 ± 0	33 ± 1	60 ± 2	63 ± 4	61 ± 0	122 ± 1	17 ± 3	24 ± 5
	C	65 ± 2	97 ± 1	84 ± 2	47 ± 2	38 ± 0	44 ± 4	80 ± 1	17 ± 1	40 ± 3	68 ± 1	45 ± 0	35 ± 0	59 ± 1	66 ± 5	61 ± 5	115 ± 1	19 ± 5	22 ± 0
	D	62 ± 3	101 ± 5	73 ± 8	50 ± 3	41 ± 2	44 ± 2	79 ± 4	19 ± 1	40 ± 0	66 ± 2	45 ± 2	38 ± 5	60 ± 2	67 ± 9	61 ± 1	120 ± 2	15 ± 3	19 ± 3
	E	64 ± 2	103 ± 1	73 ± 1	47 ± 3	38 ± 0	43 ± 3	77 ± 2	18 ± 1	41 ± 0	70 ± 6	45 ± 3	38 ± 2	62 ± 2	64 ± 7	61 ± 2	122 ± 4	14 ± 0	19 ± 1
	F	66 ± 2	94 ± 1	73 ± 7	51 ± 2	46 ± 0	41 ± 1	75 ± 1	18 ± 0	42 ± 1	73 ± 2	47 ± 1	39 ± 1	57 ± 0	57 ± 2	64 ± 4	117 ± 6	16 ± 1	23 ± 2
	G	65 ± 3	94 ± 8	77 ± 5	52 ± 3	39 ± 3	41 ± 0	76 ± 1	17 ± 3	45 ± 7	71 ± 2	50 ± 2	40 ± 1	56 ± 2	54 ± 4	75 ± 12	114 ± 9	13 ± 1	22 ± 3
	H	66 ± 1	107 ± 4	75 ± 5	46 ± 3	31 ± 6	43 ± 1	76 ± 1	18 ± 1	39 ± 3	70 ± 2	46 ± 2	37 ± 0	58 ± 0	61 ± 12	62 ± 4	124 ± 2	17 ± 3	23 ± 2
	I	62 ± 2	100 ± 2	71 ± 8	45 ± 2	38 ± 8	41 ± 3	76 ± 3	18 ± 2	41 ± 3	69 ± 1	46 ± 4	40 ± 4	62 ± 2	67 ± 3	63 ± 0	127 ± 6	15 ± 3	19 ± 1
	J	57 ± 4	99 ± 6	75 ± 1	50 ± 0	32 ± 7	40 ± 3	81 ± 7	16 ± 1	36 ± 5	77 ± 8	47 ± 0	37 ± 4	64 ± 1	59 ± 5	62 ± 1	134 ± 4	16 ± 1	17 ± 2
	K	65 ± 0	99 ± 6	79 ± 10	49 ± 1	34 ± 3	44 ± 4	78 ± 0	19 ± 3	41 ± 2	68 ± 1	44 ± 1	36 ± 3	61 ± 3	61 ± 12	66 ± 3	122 ± 2	14 ± 2	21 ± 7
	L	65 ± 1	104 ± 4	66 ± 4	53 ± 2	36 ± 3	46 ± 2	76 ± 1	19 ± 3	37 ± 2	67 ± 2	41 ± 2	38 ± 0	66 ± 2	70 ± 1	64 ± 3	120 ± 0	14 ± 0	17 ± 0
	M	68 ± 3	104 ± 4	72 ± 3	47 ± 3	36 ± 3	43 ± 1	77 ± 4	17 ± 1	39 ± 4	71 ± 0	46 ± 4	37 ± 1	61 ± 1	64 ± 11	58 ± 11	124 ± 9	16 ± 2	20 ± 0
	Entomology code	**ala**	**asp + asn**	**arg**	**gly**	**his**	**ile**	**leu**	**met**	**phe**	**pro**	**ser**	**thr**	**val**	**lys**	**tyr**	**glu + gln**	**trp**	**cys**
AUTUMN	A	64 ± 2	83 ± 1	82 ± 12	59 ± 4	41 ± 2	44 ± 2	83 ± 3	21 ± 2	51 ± 3	73 ± 2	50 ± 9	40 ± 8	66 ± 3	55 ± 7	76 ± 0	87 ± 2	19 ± 0	23 ± 1
	B	68 ± 3	91 ± 8	77 ± 3	61 ± 1	42 ± 5	44 ± 1	77 ± 3	20 ± 0	50 ± 1	74 ± 3	56 ± 2	42 ± 4	66 ± 2	47 ± 1	73 ± 2	90 ± 4	16 ± 2	21 ± 3
	C	64 ± 1	94 ± 3	77 ± 6	57 ± 2	38 ± 3	45 ± 0	81 ± 1	20 ± 2	48 ± 6	69 ± 5	58 ± 4	42 ± 5	68 ± 1	66 ± 8	68 ± 8	86 ± 1	14 ± 2	17 ± 1

	D	68 ± 2	79 ± 11	86 ± 10	61 ± 7	37 ± 1	44 ± 2	78 ± 5	20 ± 1	49 ± 5	81 ± 6	48 ± 8	37 ± 2	64 ± 3	47 ± 6	78 ± 5	97 ± 14	17 ± 0	24 ± 2
	E	66 ± 2	89 ± 4	78 ± 5	58 ± 6	39 ± 4	48 ± 2	81 ± 3	19 ± 1	48 ± 4	76 ± 3	55 ± 2	40 ± 2	67 ± 2	50 ± 11	71 ± 7	90 ± 1	13 ± 5	21 ± 0
	F	69 ± 3	95 ± 9	68 ± 6	55 ± 2	35 ± 2	48 ± 1	89 ± 3	19 ± 1	42 ± 1	69 ± 1	56 ± 5	43 ± 4	69 ± 2	63 ± 8	62 ± 4	96 ± 6	13 ± 1	15 ± 0
	G	62 ± 5	89 ± 5	75 ± 8	57 ± 4	38 ± 4	50 ± 1	79 ± 1	18 ± 2	44 ± 2	77 ± 4	51 ± 4	40 ± 2	67 ± 4	51 ± 4	69 ± 6	101 ± 10	15 ± 1	19 ± 2
	H	63 ± 2	95 ± 26	83 ± 13	55 ± 2	42 ± 2	42 ± 2	73 ± 1	20 ± 0	46 ± 1	72 ± 2	56 ± 11	37 ± 2	61 ± 1	54 ± 5	70 ± 3	102 ± 2	16 ± 1	21 ± 3
	I	68 ± 2	93 ± 2	73 ± 6	56 ± 1	40 ± 2	47 ± 1	84 ± 1	19 ± 1	46 ± 3	70 ± 0	55 ± 6	43 ± 1	67 ± 3	55 ± 6	67 ± 1	88 ± 2	15 ± 1	18 ± 0
	J	66 ± 4	85 ± 4	80 ± 3	55 ± 2	35 ± 1	43 ± 3	74 ± 1	18 ± 1	43 ± 2	79 ± 1	49 ± 3	35 ± 1	59 ± 2	60 ± 10	72 ± 1	113 ± 0	18 ± 1	23 ± 1
	K	76 ± 7	99 ± 2	70 ± 6	53 ± 1	37 ± 2	45 ± 2	79 ± 1	18 ± 0	43 ± 2	68 ± 1	53 ± 0	44 ± 8	66 ± 0	59 ± 7	60 ± 1	102 ± 6	15 ± 1	19 ± 1
	L	64 ± 5	76 ± 8	88 ± 7	55 ± 2	39 ± 2	42 ± 1	73 ± 1	19 ± 2	48 ± 5	81 ± 3	46 ± 1	38 ± 2	64 ± 2	51 ± 9	70 ± 6	107 ± 1	18 ± 0	26 ± 2
	M	65 ± 1	83 ± 16	73 ± 6	62 ± 6	40 ± 2	49 ± 4	86 ± 2	18 ± 2	46 ± 4	76 ± 4	55 ± 0	44 ± 3	72 ± 0	49 ± 9	71 ± 6	87 ± 11	14 ± 1	17 ± 0
	N	65 ± 3	85 ± 13	77 ± 11	57 ± 3	43 ± 7	43 ± 1	79 ± 5	19 ± 0	47 ± 3	73 ± 1	55 ± 5	42 ± 6	67 ± 1	56 ± 5	69 ± 1	92 ± 3	16 ± 2	20 ± 1
	O	68 ± 2	89 ± 11	73 ± 5	58 ± 4	40 ± 3	46 ± 1	80 ± 2	18 ± 1	47 ± 1	74 ± 2	54 ± 4	42 ± 1	69 ± 0	51 ± 1	68 ± 2	88 ± 1	17 ± 2	18 ± 1
	Entomology code	**ala**	**asp + asn**	**arg**	**gly**	**his**	**ile**	**leu**	**met**	**phe**	**pro**	**ser**	**thr**	**val**	**lys**	**tyr**	**glu + gln**	**trp**	**cys**
CONTROL	CTR	66 ± 2	99 ± 9	77 ± 9	51 ± 1	37 ± 1	44 ± 2	74 ± 4	18 ± 0	43 ± 3	69 ± 0	47 ± 2	39 ± 0	62 ± 3	62 ± 6	64 ± 3	109 ± 5	18 ± 2	21 ± 3

An explorative Principal Component Analysis (PCA) was performed to assess if and how different rearing substrates would affect amino acid composition. Principal Components are new variables obtained by linear combinations of the original variables (amino acids and total protein), allowing us to describe the system variability using only a few components, thus reducing the complexity, enabling the visualization of the samples in a two-dimensional graph. The analysis showed that about 39% of the total variation is explained by the first component (PC1), 57% by the first two components and 69% by the first three components. The most important variables for each principal component are reported in Table 2.8.

Table 2.8: PCA loadings.

	PC1	PC2	PC3	PC4	PC5
glu	-0,94	-0,14	-0,01	-0,20	0,04
lys	-0,90	0,16	0,08	0,19	-0,13
phe	0,86	-0,08	-0,34	0,08	0,03
asp	-0,84	0,33	-0,30	0,05	0,09
tyr	0,82	-0,31	-0,23	-0,07	-0,11
gly	0,80	0,23	-0,18	-0,30	0,26
val	0,67	0,53	0,30	0,02	0,07
ser	0,62	0,46	-0,30	-0,17	-0,19
his	0,52	-0,36	0,23	0,31	-0,48
cys	0,22	-0,82	0,22	0,08	0,06
arg	0,38	-0,76	-0,16	-0,01	-0,13
thr	0,43	0,62	-0,39	0,22	-0,14
trp	0,14	-0,53	0,00	0,47	0,50
leu	0,54	0,15	0,67	0,15	-0,22
met	0,54	0,05	-0,60	0,33	0,23
ile	0,47	0,45	0,50	0,15	0,03
pro	0,50	-0,24	0,25	-0,70	0,20
ala	0,17	0,30	0,48	0,18	0,50

Figure 2.4a shows the scatter plot of the scores of PC1 versus PC2. The loadings for the first two components are schematized in the component plot (Figure 2.4b). Glutamic acid/glutamine, lysine, phenylalanine, aspartic acid/asparagine, tyrosine, glycine, valine, serine and histidine turned out to be the most influencing variables for PC1, PC2 was predominantly characterized by cysteine, tryptophan, arginine and threonine, while leucine, isoleucine and methionine had the greatest effect for PC3. Figure 2.4a shows a partial separation of the BSF prepupae samples, based on the seasonality of the substrates. In particular, BSF prepupae that had been reared on substrates belonging to the Autumn group are found in correspondence with PC1 positive values, well separated from the others. In this group, the most represented essential amino acids are phenylalanine/tyrosine, valine and leucine. On the other hand, the All-Year and Summer groups showed a less clear separation between each other, and both were found at negative values of PC1. A less clear separation between each other and both were found at negative values of PC1. They differed from the Autumn group mainly in their higher amounts of lysine, aspartate and glutamate content. Finally, the control group was found in an intermediate position, closer to the All-year and Summer groups and with greater differences compared to the Autumn one.

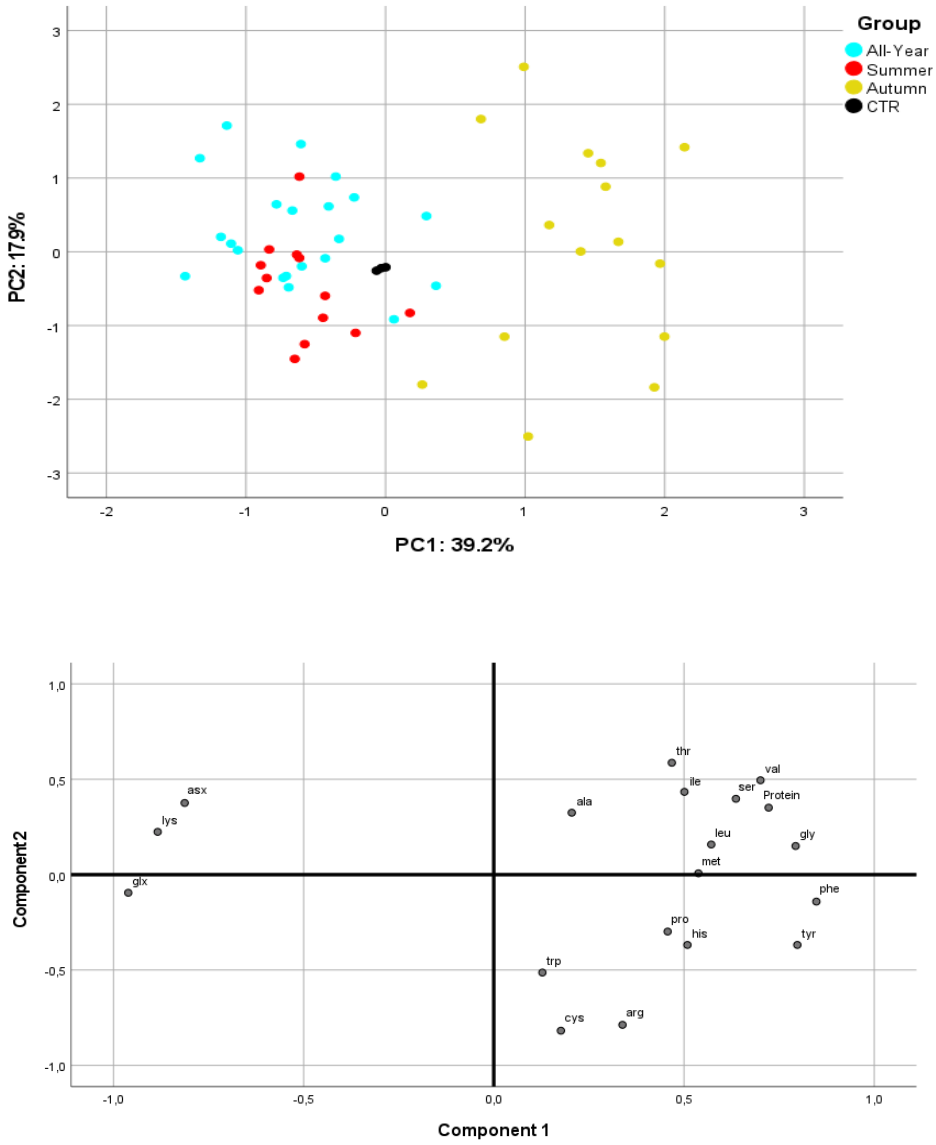

Figure 2.4 (a) Score plot of BSF samples on the first two principal components; (b) loadings values of the variables associated with the first two principal components.

In order to verify to what extent, the nutritional properties of BSF prepupae proteins were affected by the composition of the rearing substrates, a one-way ANOVA was carried out on the essential amino acids (EAAs), dividing the samples according to the four groups of substrates (Table 2.9). As a general consideration, ANOVA results confirm what is evidenced by PCA; in fact, the Autumn group is the one presenting the larger number of significant differences with respect to the other experimental diets (All-Year and Summer), and also with respect to the control diet. The BSF prepupae of the Autumn group contain the highest amounts of essential amino acids, except for lysine, which was detected in significant lower amounts compared to the BSF reared on the other substrates. Isoleucine, methionine, cysteine and tryptophan did not differ significantly in any of the four diets. On the other hand, specific differences in essential amino acid content of prepupae reared on the three diets were observed. Leucine turned out to be the EAA most affected from the diet, highlighting a sharp decline when samples of the All-Year group were considered. A similar trend was also observed for valine and histidine. Phenylalanine and tyrosine were lower in the All-Year and Summer groups when compared with the Autumn group, while threonine in the Summer group was present in a lower amount with respect to the Autumn group. Actually, several peculiar differences were also observed in the single samples (Table 2.7).

Table 2.9. Mean values (expressed as mg/g protein) for essential and semi-essential amino acids of BSF prepupae that had been reared on the different groups of substrates.

	ALL-YEAR (n=21)	**SUMMER (n=13)**	**AUTUMN (n=15)**	**CTR (n=3)**
His	33.50 a	37.86 ab	39.00 b	37.00 ab
Ile	42.89 a	43.11 a	44.10 a	45.39 a
Leu	71.24 a	77.66 bc	79.88 c	74.00 ab
Val	60.49 a	60.25 a	66.09 b	62.43 a
Lys	62.22 a	62.93 a	51.73 b	62.44 a
Cys	18.17 a	20.42 a	20.14 a	20.70 a
Met	18.37 a	17.54 a	19.16 a	18.40 a
Phe	41.69 a	40.03 a	46.47 b	42.53 ab
Tyr	61.45 a	63.16 ab	69.67 b	64.13 ab
Thr	39.50 ab	37.36 a	40.58 b	39.30 ab
Trp	14.80 a	15.76 a	15.84 a	17.60 a

Values followed by different letters within one row are significantly different (one-way ANOVA, Tukey post hoc test, $p < 0.05$).

Our results suggested a partial influence of the BSF diet on the total protein production and on their amino acid composition, and similar findings were also obtained by Spranghers et al. (2017) and Soetemans et al. (2020) on the tenebrionid *Alphitobius diaperinus* (Soetemans et al., 2020; Spranghers et al., 2017). However, it is not yet clear how BSF larvae convert the amino acids from the diet into amino acids useful for their metabolism and how they store and use differently the different essential amino acids. According to Liland et al. (2017) BSF larvae were able to produce certain amino acids, such as tyrosine, which were almost absent in the seaweed-containing media (Liland et al., 2017). Due to the complexity of the system and the possible interaction of diet components on specific essential amino acid content, a multivariate statistical analysis was used as further approach to better understand the influence of the diet composition (Table 2.6) on the essential amino acid profile. This approach was possible because the experimental diets

were formulated according to a Design of Experiments (DoE) (Liland et al., 2017). As mentioned above, this makes it possible to define the correlation among food leftovers' composition and the resulting amino acid content of prepupae. The interaction results, in terms of statistical significance (ANOVA) are showed in Table 2.10. Here it is possible to see the influence of many significant factors for each response, thus confirming the complex nature of these correlations. These relationships are in general better explained by the interaction between the factors rather than by an independent single factor, confirming the need for the multivariate approach. Furthermore, the fitting parameters show a fairly good fitting (R2 > 0.50) only for some responses, in particular for leucine, valine and lysine (Table 2.10).

Table 2.10. Summary of ANOVA results

Response	Transformation	Significant factors other than linear factors	R^2	Pred R^2
		Ashes*Fibers*Carbohydrates	0.39	0.25
	None	Ashes*Fibers		
		Ashes*Carbohydrates		
		Fibers*Carbohydrates		
his	= 115,1251 * Lipids + 142,8697 * Ashes + 38,8390 * Fibers – 1685,9516 * Polyphenols + 43,4625 * Proteins + 39,3171 * Carbohydrates -250,6740 * Ashes * Fibers -272,3182 * Ashes * Carbohydrates – 9,7396 * Fibers * Carbohydrates + 506,4663 * Ashes * Fibers * Carbohydrates			
	None	Ashes* Proteins	0.42	0.34
ile	= 74,8870 * Lipids – 40,7818 *Ashes + 43,3120 * Fibers -1796,7832 * Polyphenols+ 37,9499 * Proteins + 46,5295 * Carbohydrates + 972,5378 *Ashes * Proteins			

leu	Power (k=2.5)	None	0.76	0.73
	= 138,8949 * Lipids + 56,4144 * Ashes + 67,6174 * Fibers + 1404,0497 * Polyphenols + 128,6415 * Proteins + 68,7274 * Carbohydrates			
met	None	None	0.38	0.30
	= -0,1543 * Lipids + 8,1371 * Ashes + 22,8876 * Fibers -1975,3666 * Polyphenols + 15,3176 * Proteins + 19,1364 * Carbohydrates			
phe	None	Lipids*Fibers	0.51	0.44
	= -40,2706 * Lipids + 20,9231 * Ashes + 44,2497 * Fibers – 8333,6909 * Polyphenols + 51,8682 * Proteins + 46,1981 * Carbohydrates + 371,7611 * Lipids * Fibers			
thr	None	None	0.47	0.41
	= 20,2967 * Lipids + 29,0736 * Ashes + 39,5218 * Fibers -3503,6628 * Polyphenols + 59,5214 * Proteins + 40,8414 * Carbohydrates			
val	None	Lipids * Carbohydrates Polyphenols * Proteins	0.68	0.62
	= 207,4451 * Lipids + 38,1122 * Ashes + 60,6184 * Fibers -11825,8780 * Polyphenols + 74,0273 * Proteins + 66,4548 * Carbohydrates -304,7381 * Lipids * Carbohydrates + 75299,2771 *Polyphenols * Proteins			

lys	Power (k=2.5)	Lipids * Ashes * Polyphenols Lipids * Ashes Lipids * Polyphenols Lipids * Carbohydrates Ashes * Polyphenols	0.63	0.52
	-789,5534 * Lipids + 67,3562 * Ashes + 63,5633 * Fibers + 5323,1377 * Polyphenols + 65,4436 * Proteins + 56,3629 * Carbohydrates + 3172,7599 *Lipids * Ashes + 244403,0963 * Lipids * Polyphenols – 455,9764 *Lipids * Carbohydrates + 18499,4861 * Ashes * Polyphenols + 4911650,4178 * Lipids * Ashes * Polyphenols			
trp	None	Polyphenols * Proteins	0.19	0.08
	= 21,8816 * Lipids + 11,1148 * Ashes + 16,4850 * Fibers + 7629,3982 * Polyphenols + 23,4770 * Proteins + 11,7696 * Carbohydrates -71645,1372 * Polyphenols * Proteins			

The graphical representation of the results with acceptable fitting quality is reported in Figures 2.5 and 2.6. According to these results, for the leucine content in the BSF prepupae (Figure 2.5), a strong interaction emerges with the content of lipids and proteins in the rearing substrate. In particular, when the rearing diets were lacking carbohydrates, the highest content of leucine was detected when the content of proteins and lipids in the substrate was equal to or above 50 wt% (Figure 2.5a). In the presence of a higher content of carbohydrates (50 wt%), the highest content of leucine in BSF prepupae is obtained with slightly lower contents of both proteins and lipids in the rearing diet (Figure 2.5b). Overall, the increase in carbohydrate content leads to a reduction in the red area, thus indicating that a smaller combination of rearing substrates is suitable for an increase in the leucine content of the BSF prepupae. In conclusion, the highest leucine content in the BSF prepupae can be achieved by increasing the amounts of protein and lipids while reducing the carbohydrates content in the rearing diet. Similarly (data not shown), the valine content

in BSF prepupae is enhanced by maximising the content of lipids and proteins and reducing that of carbohydrates in the rearing substrate. The correlation between the content of leucine and valine in the BSF prepupae with the protein content of the rearing substrates could indicate that these amino acids are essential for BSF development. Finally, the contour plots related to the content of lysine in BSF prepupae (Figure 2.6) show that carbohydrate variation plays a crucial role in enhancing this amino acid content. In fact, the highest content of lysine in the insects can only be obtained when the rearing substrate has at least 90% DM of carbohydrates (Figure 2.6b) and a slightly higher amount of lipids compared to protein and fibre. A rearing substrate based on 75% DM of carbohydrates (Figure 2.6a) will result in prepupae with lower amounts of lysine. In any case, to obtain average values of lysine in the BSF prepupae, in both situations a well-balanced amount of protein and fibre in the rearing substrate is needed.

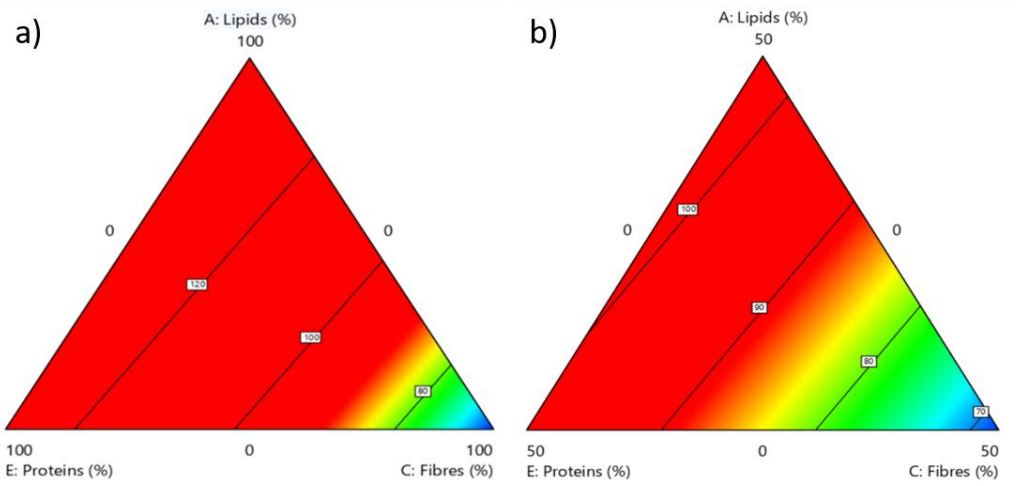

Figure 2.5 Graphical model variation of the amount of leucine in the proteins of BSF prepupae in relation to the composition of the rearing substrate in terms of lipids, proteins and fibers, considering two scenarios: (a) carbohydrates = 0 wt%; (b) carbohydrates = 50 wt%. The region representing the highest value of the response is shown in red colour whereas the lowest values are in blue. For each response, the most significant factor has been considered for the graphical model, expecting a higher variation in the response behaviour.

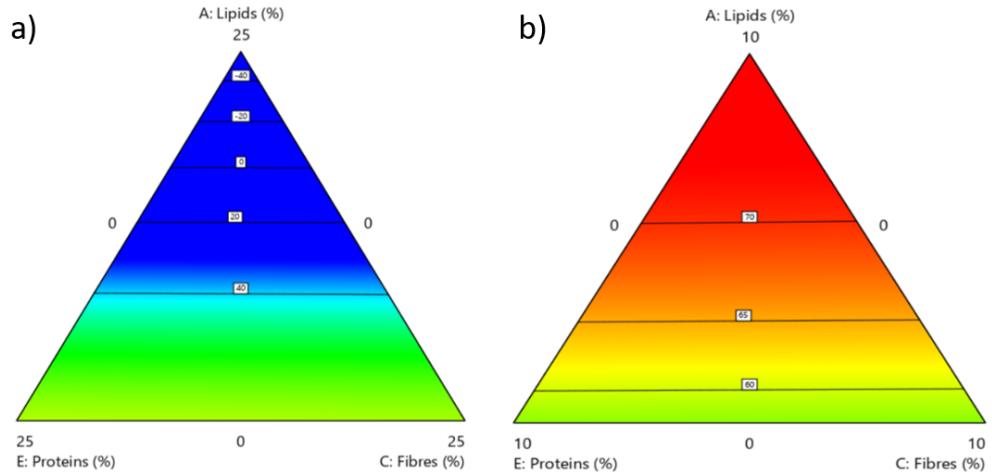

Figure 2.6 Graphical model variation of the amount of lysine in the proteins of BSF prepupae in relation to the composition of the rearing substrate in terms of lipids, protein and fibre, considering two scenarios: (a) carbohydrates = 75 wt%; (b) carbohydrates = 90 wt%. The region representing the highest value of the response is shown in red colour, whereas the lowest values are in blue. For each response, the most significant factor has been considered for the graphical model, expecting higher variation in the response behaviour.

As a general consideration, it is important to highlight that none of the BSF prepupae reared on experimental diets contained significantly lower amounts of essential amino acids with respect to the control group, except in the above-mentioned case of lysine. Despite the specific differences, the majority of the proteins obtained from the BSF prepupae reared on the experimental substrates contain on average a sufficient quantity of each EAA required for human consumption, as shown in Table 2.11. Recent studies have shown that BSF prepupae contain optimal amounts of all the essential amino acids (EAA) to satisfy the human adult requirements, as established in the reference of FAO/WHO (Caligiani et al., 2018; Cappellozza et al., 2019; De Marco et al., 2015).

Table 2.11. Highest, lowest and average essential amino acid content of BSF prepupae, compared with the FAO protein reference for human adults (2011).

	Reference protein FAO 2011 (mg/g protein)	Average values found in BSF prepupae protein (mg/g protein)	Lowest values found in BSF prepupae protein (mg/g protein)	Highest values found in BSF prepupae protein (mg/g protein)
His	16	36	23	46
Ile	30	44	39	50
Leu	61	76	67	89
Lys	48	58	47	72
Cys + Met	23	38	32	45
Phe + Tyr	41	107	91	127
Thr	25	39	33	44
Trp	6,6	15	7	19
Val	40	62	56	72

According to these figures, all the analysed BSF prepupae can meet the FAO requirements, and, as in the case of histidine and tryptophan, the EAA content is even double the required amount. Moreover, the lowest values found in the samples turned out to be higher than the recommended amounts, except for lysine, which resulted in being at the lower limit in few cases (two of the BSF samples reared on the Autumn substrates). This finding is particularly relevant, considering that the analysed prepupae samples did not undergo any thermal treatment, which, on the other hand, is very likely to occur when BSF proteins are to be used in food and feed formulations, thus further lowering the lysine amount through the Maillard reaction. Moreover, one factor that could affect the lysine content is the killing method for BSF prepupae (Leni, Caligiani, et al., 2019). Killing by freezing, which was the method used in this experimental plan, leads to some alteration of the total amino acid fraction, with the notable decrease of lysine and cysteine, likely involved in the process of melanisation, reacting with quinones with their side chain. In conclusion, although some of the experimental diets seemed to yield an increased protein fraction of the prepupal

biomass, in all cases the total amino acid profile preserved its high nutritional quality. This finding highlights that BSF prepupae are able to grow on all kind of organic residues, no matter the starting composition, always yielding acceptable protein fractions. Thus, BSF rearing seems an efficient way to dispose agrifood leftovers, and also to convert low value substrates in a high-quality protein biomass.

2.4 Conclusions

Due to its physiological characteristics and excellent nutritional properties, BSF is considered one of the most promising candidates in insect farming for feed and food purposes. Aiming at evaluating the effect of the nutrients of the rearing substrate on the nutritional content focusing on protein content and composition of BSF prepupae, we examined 49 different substrates that consisted of variable proportions of different vegetable by-products and were divided into three groups according to their seasonal availability. Focusing on protein fraction, the results showed that the total protein content of BSF prepupae ranged between 35% and 49% DM, with the highest values in the Autumn group substrates. It has also been observed that a higher protein content in the diet resulted in a higher prepupae protein content up to a certain value; this vegetable-to-animal protein conversion lowered gradually as dietary proteins were increased, indicating the existence of a minimal critical amount of protein that has to be present in the diet, and that once reached makes any further protein addition to the rearing substrate much less relevant. Dietary fibers also seem to play a positive role in the achievement of BSF prepupae protein biomass. An important outcome of this work focuses on the essential amino acids. Higher amounts of essential amino acids in the BSF prepupae that had been reared with the substrates of the Autumn group were observed. Lysine, leucine and valine were found to be the most correlated with the presence of nutrients of the feeding diet. Leucine and valine were strongly dependent on the content of protein and lipid in the diet, while lysine is correlated to the amount of carbohydrates. Despite these important differences, the essential amino acid composition almost always fully satisfied the FAO nutritional requirements for humans. The only exception was lysine, and only in a very

limited number of cases. In conclusion, this study shows that by providing BSF larvae with substrates based on a very wide range of combinations of vegetable by-products, it is possible to obtain in the BSF prepupae a protein quality very similar to the one obtained with the control diet. However, when the employed leftovers have a very low-quality nutritional content, the development of BSF biomass is less efficient, and as a consequence a lower amount of protein is obtained. Thus, in terms of maximum yield of protein obtainable from the prepupae rearing, the growing substrate formulation should be performed by carefully balancing the ì leftovers in order to avoid the disadvantage of having a slightly lower amount of protein. Anyway, if the ultimate aim is to have a biological and efficient method of reducing agricultural residues and converting them into biomass with a higher protein value than the original one, then it is no longer necessary to make a specific selection of substrates on which to grow insects since, as seen above, in all cases leftovers are convertible into a protein biomass that has a satisfactory amino acid profile. The prepupae have proven in fact to be capable of valorizing all the organic residues supplied to them, even those with the lowest initial nutritional composition. These findings are very important in view of promoting BSF as a flexible tool able to bio-convert a low protein value vegetable by-product which can vary according to seasonality or areas of production, to a high protein value biomass.

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CHAPTER 3

Development of a quantitative UPLC-ESI/MS method for the simultaneous determination of the chitin and protein content in insects.

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Anna Valentina Luparelli, Giulia Leni, Andrea Fuso, Sara Palini, Stefano Sforza, Augusta Caligiani. Development of a quantitative UPLC-ESI/MS method for the simultaneous determination of the chitin and protein content in insects.

Abstract

In a context where the commercial interest in insect chitin is always increasing, an accurate and precise method to quantify this biopolymer is required. Moreover, quantification of insect crude protein through nitrogen determination is normally overestimated due to the presence of chitin. In this work, for the first time, an RP-UPLC-ESI/MS method for the simultaneous quantification in insects of chitin, as glucosamine, and protein content, as total amino acids, is presented. The method is based on acid hydrolysis and derivatization of amino acids and glucosamine with the AccQ Tag reagent. Method was optimized and validated in terms of linearity, LOD and LOQ, intraday and inter-day repeatability, and accuracy. A hydrolysed commercial chitin was used as reference standard. The instrumental LOD and LOQ corresponds respectively to a concentration of 0,00068 mM and 0,00204 mM. The intraday precision satisfied the Horwitz ratio. Data from inter day precision showed the necessity to perform the analysis within one week utilizing standard calibration solutions freshly prepared. A matrix effect, which suggested the necessity to use an internal calibration curve or to work in a particular concentration range of glucosamine. The chitin and protein content in Black Soldier Fly and Alphitobius diaperinus were found in agreement with results obtained by independent methods. The optimized method was also tested on two different commercial food supplements, suggesting its applicability on a wide range of matrices. This newly developed method proved to be simple, more accurate and faster if compared to methods which separately analyse chitin and protein content.

3.1 Introduction

The scientific interest in polysaccharides from natural origin, in the last decade, is growing considerably thanks to their many beneficial properties observed by different studies: possible antitumoral, antioxidant, immunomodulatory, anti-inflammatory activities were in-fact suggested for polysaccharides isolated by plants, fungi, seaweed and animals (Yu et al., 2018). Chitin is one of the most abundant polysaccharides presents in nature. It is a large and linear structural polymer composed of repeating N-acetyl-D-glucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose) units, β (1→4) linked together, principally found in cell wall of fungi and bacteria, in crustaceans exoskeleton, in insect cuticle, and in internal structure of other invertebrates (Muthukrishnan et al., 2012). The interesting properties of chitin such as biodegradability, biocompatibility with living tissue, low toxicity, robustness, film forming capability allow the application of this polysaccharide and its derivatives in a wide range of sectors (Martínez et al., 2014). In biomedical area, chitin derivatives, especially chitosan (chitin deacetylated form), are applied as antimicrobial, antiviral, antitumor, antioxidant, antihypertensive, anti-inflammatory agents. Thanks to its robustness, chitosan is also fruitfully used as biomaterial for tissue engineering and regenerative medicine (Kumar et al., 2004; Wan & Tai, 2013). The constituent unit of chitin, glucosamine, is instead often added to human supplements against osteoarthritis (Nakamura, 2011; Reginster et al., 2001). The pharmacological interest potentialities of chitin as drug carrier are also important. In fact, its chemical structure offers links mechanisms which allow the carrying and controlled release of active components of drugs and non-viral genes (Janes et al., 2001; Prabaharan, 2008). Chitin and derivatives also found multiple applications in food and agriculture sector: the amino sugars capacity to electrostatically interact with lipids make them perfect for use as ingredients in nutritional supplements for weight loss, and human cholesterol control, to be used sparingly because of significant downsides on minerals and liposoluble vitamins absorption levels. (Koide, 1998; Muzzarelli & Muzzarelli, 2006; Razdan & Pettersson, 1994). Even if the mode of action is not well understood yet, it is also well known that chitinous compounds can inhibit the growth of pathogens. This anti-microbial power makes them

perfect as food preservatives and against plant fungal pathogens in agriculture (Shahidi et al., 1999). Chitin potentialities are still being discovered, especially in emerging sectors, such as nanobiotechnology, in which chitin compounds can found a role in promising future applications as low-cost and biodegradable nanocomposite polymers (Khoushab & Yamabhai, 2010). Currently, chitin available on the market and used for industrial applications comes mainly from processing waste of crustaceans (Brigode et al., 2020). These chitinous discards can easily reach 20 million tons por year (FAOSTAT, 2001). However, due to the increasing market interest for this biopolymer, new natural and sustainable sources of chitin, able to meet its growing industrial demand, are being investigated (Brück et al., 2011; Morganti & Chen, 2015). In this context, insect chitin would represent an excellent alternative to the crustacean chitin. In all the insect species, chitin is present as main component of the cuticular exoskeleton, where it plays a structural and defensive role. It also provides support and protective function in some insect internal structures such as the intestinal canal, trachea, genital ducts, and salivary glands (Muthukrishnan et al., 2012; K. Y. Zhu et al., 2016). Several studies carried out to evaluate the nutritional composition of edible insects showed that the insect chitin content ranges from 11,6 to 137,2 mg per kg of dry mass (Finke, 2007; Kouřimská & Adámková, 2016). Chitin from insects is beginning to be considered as an advantageous alternative to chitin from other sources, thanks to the recent attention in the use of insects as sustainable protein sources for the increasing food and feed demand. Indeed, the perspective of increasing insect biomasses production for protein extraction will lead to a high availability of insect chitin (Cortes Ortiz et al., 2016). However, the chitin high molecular weight, its insolubility in water and most other solvents and the peculiar association that occur between cuticular proteins and chitin during the hardening process of the insect exoskeleton make the biopolymer isolation and characterization very difficult (Brigode et al., 2020; Ishimaru et al., 2016; Rudall, 1963).

One of the most challenging problem is the quantification of chitin in insect material and other biomasses. Many direct and indirect methods, used to measure the chitin fraction in insects, bear indeed some limitations. Weighing the chitin residue after removal of lipids,

proteins, and minerals is a very time-consuming approach. An indirect gravimetric determination of insect chitin based on the measurement of the acid detergent fibre (ADF) has been described by Finke (Finke, 2007), but this analysis is specific for other type of compounds (non-acid-labile) and the total amino acids need to be subtracted from the insoluble residue obtained, hampering the determination precision. Another indirect approach generally used in biological samples is measuring acetyl groups released from chitin after acid hydrolysis (Finke, 2007; Hahn et al., 2018; Han & Heinonen, 2021). In addition to being a long analysis, the polymer initial acetylation degree is to be exactly known, otherwise the final value obtained cannot be considered accurate (Finke, 2007; Hahn et al., 2018; Han & Heinonen, 2021). Direct methods are generally preferred: the chitin monomer glucosamine (GlcN) released after an acid treatment of the sample (X. Zhu et al., 2005) can be determined by colorimetric (Bierstedt et al., 1998; W. Chen & Chiou, 1999; Frey et al., 1994; Nitschke et al., 2011), capillary electrophoresis (Jáč et al., 2008; Volpi, 2009), or chromatographic (Han & Heinonen, 2021; X. Zhu et al., 2005) (gas or liquid chromatography) methods. Indeed, to date, quantifying chitin through the glucosamine released after hydrolysis in strong acid environment is the best way to get fairly accurate chitin values in the insect samples. Anyway, to improve the capacity of measuring chitin correctly, the direct determination methods are subjected to continuous optimizations (Hahn et al., 2018; Sanches-Silva et al., 2012). However, some weakness points remain: the long analysis times, the large use of chemical agents, the insufficient sensitivity of the method and the need to remove the sample impurities or protein fraction are the biggest limitations (Han & Heinonen, 2021). Chitin has also been previously evaluated by determining the total nitrogen content and subtracting the protein contribution determined by total amino acid analysis (Caligiani et al., 2018). However, also this method is quite long, and provides only an indirect measure of chitin content.

The uncertainty in chitin content can also affect the protein determination when made through nitrogen-measuring methods (Dumas or Kjeldahl), since the nitrogen-containing chitin molecules lead to an overestimation of the protein content.

In view of all the factors that may hinder advance towards a correct molecular characterization of insects, and other chitin-containing biomasses, a faster and more precise quantification method of insect chitin, and proteins, is needed. This work aims to develop and validate a UPLC/ ESI-MS method for simultaneous quantification of amino acids and glucosamine, released from insect proteins and chitin respectively, after total acid hydrolysis.

To the best of our knowledge, this is the first time that a method is developed with the aim to simultaneously determine glucosamine and total amino acids by using UPLC/ESI-MS.

3.2 Materials and methods

3.2.1 Chemicals

Kjeldahl defoamers and catalyst were purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit and AccQ-TagTM were obtained from Waters (Milford, MA, USA). DL-norleucine, amino acid standard mixture, Chitin from shrimp shell (practical grade), Chitin from shrimp shell (purified powder), D-(+)-glucosamine hydrochloride, D-(+)-N-acetylglucosamine, D-(+)-galactosamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of the practical grade and purified powder chitin from shrimp shell was determined according to the Kjeldahl analysis based on the measurement of chitin nitrogen. Assuming a fully acetylated chitin, the conversion from nitrogen content to amount of chitin was made using the specific nitrogen conversion factor calculated for chitin 14,5 (Caligiani et al., 2018). The purity was found to be 89,3% ± 2,4 for the commercial practical grade chitin and 101,5% ± 1,5 for the commercial purified powder chitin. All the other solvents, salts, acids, and bases were of analytical grade and purchased from Sigma-Aldrich or Carlo Erba (Milan, Italy).

3.2.2 Insect and food supplement samples

Black soldier flies (*Hermetia Illucens,* HI) at prepupae stage were obtained from a colony reared in the laboratory of Applied Entomology of the University of Modena and Reggio

Emilia (Italy) since 2016, established starting from larvae purchased from CIMI srl (Cuneo, Italy).

Lesser Mealworm (*Alphitobius diaperinus*, AD) larvae were provided by Protifarm (Ermelo, The Netherlands). Both insect species were killed by freezing at -20 °C. Frozen insects were ground for 2 minutes with IKA A10 laboratory grinder (IKA Werke GmbH & Co. KG, Staufen, Germany). After grinding samples were immediately used for the analysis or stored at -20°C until use. Additionally, two different commercial dietary supplements containing polymers of glucosamine, were bought on the market.

3.2.3 Preparation of N-acetyl glucosamine, glucosamine, galactosamine, chitin and amino acids standard working solutions.

For the amino acid determination, external calibration was performed as previously reported by Caligiani et al, 2018, using standard solutions prepared by mixing 20 µl of 5 mM Norleucine in HCl 0.1 M as reference standard, with different amounts of amino acids standard mixture (2,5 mM) to obtain five concentrations (1.2, 0.75, 0.5, 0.25, 0.1 mM).

In the case of chitin quantification, 4 different standard solutions were tested: i) a standard glucosamine hydrochloride solution, to be used as such, at a final concentration of 2,5 mM, prepared by dissolving 50 mg of glucosamine hydrochloride in HCl 0,1 M up to a final volume of 100 mL. ii) a standard solution of glucosamine subjected to acid hydrolysis, iii) a standard solution of N-acetyl glucosamine subjected to acid hydrolysis and iv) practical grade chitin after acid hydrolysis. Solutions ii), iii) and iv) were prepared by dissolving respectively 50mg, 50mg and 30mg of corresponding analyte in 6 mL of hydrochloride acid solution (6N) and hydrolysed (23h, 110°C) following the same conditions set out for protein hydrolysis (see the next paragraph) and used for the sample preparation, then brought to volume of 100 mL with distilled water, reaching a final concentration of 2,5 mM for hydrolysed glucosamine and N-acetyl glucosamine and of 1.65 Mm (expressed as glucosamine) for hydrolysed practical grade chitin. All these stock solutions were diluted with Milli-Q water, in order to obtain from each one, five working solutions having 1.5, 1, 0.75, 0.5, 0.25 mM final concentration of glucosamine. Before making the individual working solutions up to volume, to each one 8 µl of galactosamine hydrochloride (46 mM)

were added as standard, prepared by adding 1 mL of HCl 0,1 M to 10 mg of galactosamine. These solutions were used to determinate the linearity of the method. The hydrolysed stock solution of practical grade chitin was also used to build the external calibration curve for the quantitative determination of chitin, for the determination of the limit of detection (LOD), and for the determination of the limit of quantification (LOQ).

3.2.4 Hydrolysis of insect and food supplement matrices

Total amino acids and glucosamine were respectively released from protein and chitin fraction of analysed samples by strong acid hydrolysis following the procedure described by Caligiani et al. (Caligiani et al., 2018), with some modifications. Approximately, 500 mg of grinded insect samples (black soldier fly prepupae and lesser mealworm larvae) and 30 mg of the commercial dietary supplements were weighed in Pyrex glass tubes, directly mixed with 6 mL of HCl 6 N and hydrolysed at 110 °C for 23 h. At the end of hydrolysis, 7.5 mL of 5 mM Norleucine in HCL 0.1 N, used as internal standard for total amino acid determination, was added after cooling of the sample. The hydrolysates were then filtered using a Buchner filter and brought up to volume of 100 mL with deionized water. Then, 450 µl of hydrolysate solution were taken and mixed with 8 µl galactosamine 46 mM. The solution was brought up to 500 µl with deionized water. The hydrolysates containing the two internal standards were stored at -20°C until derivatization.

3.2.5 Derivatization

The derivatization of the hydrolysed sample was necessary to detect total amino acids and glucosamine content by reversed phase UPLC. It was performed according to the method described by Leni et al. (Leni, Caligiani, et al., 2019). 70 µl of borate buffer, 10 µl of hydrolysed samples or standard solutions, 20 µl of reconstituted AccQ Tag reagent (Waters Co., Milford, U.S.A.) were mixed and kept at 55°C for 10 minutes. To complete the derivatization, the mixture was diluted with 300 µl of Milli-Q-water and mixed well. After dilution, the solution was put into an LC vial for UPLC-ESI/MS analysis.

3.2.6 UPLC-ESI/MS analysis of Amino Acids and Glucosamine

For glucosamine and amino acids derivatives, separation was carried out by UPLC/ESI-MS analysis, performed by using a Waters ACQUITY Ultra Performance LC system with an Acquity BEH C18 column (1.7 µm, 2.1x150 mm). The chromatographic conditions were the same already described by Caligiani et al., 2018 (Caligiani et al., 2018) for amino acids determination in insect samples with some modifications: mobile phase was composed by H_2O + 0.2 % CH_3CN + 0.1 % HCOOH (eluent A) and CH_3CN + 0.1 % HCOOH (eluent B). Gradient elution was performed: isocratic 100 % A for 1.8 min, from 100 % A to 50 % A by linear gradient in 11.4 min and 0.8 min at 50 % A plus washing step at 0 % A (100 % B) and reconditioning. Flow rate was set at 0.20 mL/min, injection volume 4 µl, column temperature 35 °C and sample temperature 18°C. Detection was performed by using Waters SQ mass spectrometer: ESI source in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 300 °C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h, full scan acquisition (100-2000 m/z), scan duration 1s.

3.2.7 Optimization and Validation of UPLC-ESI/MS Method for Chitin quantification

3.2.7.1 Preparation of insects spiked samples.

Five different samples containing five hundred of milligrams of grinded whole black soldier fly prepupae were spiked with different amounts of standard practical grade chitin to obtain final added concentrations of glucosamine of 1.5, 1, 0.75, 0.5 and 0.25 mM. The experiment was carried out in triplicate. Spiked samples were then subject to the same hydrolysis and derivatization protocol described in the paragraphs 3.2.4 and 3.2.5. These samples were used to build an internal calibration curve, utilized to obtain the real amount of chitin in the prepupae sample, corresponding to the X intercept value, and also for the evaluation of an eventual matrix effect, by comparing the internal calibration with the external calibration curve. The global recovery rate was calculated by comparing the determined amount of chitin in the spiked samples to the amount added. The amount of chitin, expressed as chitin% DM, was calculated by using the weighted mass (g) of insect

sample and the added mass (g) of practical grade chitin, assuming 100% release of GlcN after hydrolysis.

3.2.7.2 Linearity and Accuracy

The linearity of the method was tested on the four different standards reported in Paragraph 3.2.3, all potential candidates to be used as external reference standards in the analysis. Five concentration levels (1,5, 1, 0,75, 0,5, 0,25 mM expressed as glucosamine concentration) were obtained for each standard: pure glucosamine hydrochloride, glucosamine hydrochloride subjected to hydrolysis protocol, N-acetyl Glucosamine subjected to hydrolysis protocol, and practical grade chitin standard subjected to the hydrolysis protocols. Two replicates were performed per each concentration of each standard. 8 microliters of galactosamine hydrochloride 46 Mm were added to each solution. A pure chitin hydrolysed standard (101,5% ±1,5 purity) solution was prepared as a control sample and used to check the accuracy of each calibration. Regression was performed on the ratio of peak areas of analyte and internal standard versus analyte concentration added. The goodness of fit was determined by means of Mandel test at the 99% significance.

3.2.7.3 Limit of Detection and Limit of Quantification.

The limit of detection and limit of quantification were calculated utilizing the S/N ratio methods, based on the determination of the peak-to-peak noise (Apostol, I., et al., 2009). LOD and LOQ were calculated as the concentrations of chitin producing after hydrolysis a recognizable glucosamine peak with a signal-to-noise ratio of, respectively, 3,3 and 10. LOD and LOQ were determined in pure standard solution of hydrolysed chitin (practical grade).

3.2.7.4 Repeatability and Effect of time on GlcN-AccQ-Tag yield under derivatization

Repeatability was evaluated as intraday and inter day precision. Intraday repeatability of the method was calculated for six different concentration levels, using the same spiked samples prepared according to the procedure explained in paragraph 3.2.7.1. The results

of intraday precision were compared with Horwitz predicted intra laboratory precision (PRSD) by the calculation of Horwitz ratio (HORRAT) (Horwitz & Albert, 2006).

Inter day repeatability was instead calculated in a four weeks' interval of time on two sets of four replicates of calibration standard (practical grade chitin) and BSF prepupa in two different storage conditions (before and after derivatization protocol). Briefly, replicates of 30 mg of practical grade chitin and of 500 mg of BSF prepupae where subjected to hydrolysis protocol as reported in the paragraph 3.2.4. 10 µL of each hydrolysed solution were then subjected to react with AccQ-Tag. After derivatization, the mixtures were inserted into an LC vial for UPLC-ESI/MS analysis and stored at -20°C until analysis. The rest of each prepared sample was stored underivatized at -20°C and derivatized immediately before the analysis. All samples were analysed every week for consequent four weeks. The results of inter day precision were compared with Horwitz predicted intra laboratory precision (PRSD) by the calculation of Horwitz ratio (HORRAT) (Horwitz & Albert, 2006), too. Data from inter day precision were also used to provide information about the stability of GlcN and GlcN-AccQ derivative during the time.

3.2.7.5 Data Analysis

Data on chitin content were obtained as triplicate analysis of independent hydrolysed samples and are presented as mean ± SD. The analyte amount was calculated using hydrolysed practical grade chitin as standard for external calibration with five concentration levels (see paragraph 3.2.7.2). In the chromatographic analysis glucosamine is present in the two anomeric forms, alpha and beta. To calculate the chitin content the sum of the areas of the two peaks was considered, according to the equation below:

$$\%\, Chitin\colon \left(\frac{Cis\ *\ PAg\ *\ MWg}{PAis\ *\ Mass}\right) * 100$$

Were Cis is concentration of internal standard in the calibration solution; Pag: peak area of glucosamine; MWg: molecular weight of glucosamine; Pais: peak area of the internal standard (galactosamine); Mass: mass in grams of the insect sample.

Due to the use of chitin concentration in the calibration curve, the values obtained are directly expressed as chitin, even if the analytical target is glucosamine.

3.3. Results and discussion

3.3.1 Development of Analytical Method for the simultaneous Quantitative Determination of Total amino acids and Glucosamine in Insect Samples.

3.3.1.1 Choice of the UPLC-ESI/MS conditions.

The UPLC-ESI/MS method conditions chosen for the simultaneous determination of total amino acids and chitin in insect samples were the same used to quantify total amino acids in BSF prepupae (Caligiani et al., 2018). This method, combined with separated determination of tryptophan and sulphurated amino acids, is widely adopted to determine the full amino acid profile of food samples and the sum of amino acids can be used also to determine accurately the total protein content in insects (Caligiani et al., 2018). Despite glucosamine from chitin is in principle detectable in the method conditions as AccQ TAG derivatives, its content in insect samples was never determined simultaneously with the amino acid content by an UPLC-ESI/MS chromatographic method. So, the aim of this work was to optimize and validate the analytical method for amino acid analysis also to directly evaluate the chitin content (via glucosamine detection) of insect samples. As a first step it was necessary to assess the suitability of the UPLC-ESI/MS parameters in separating it. For this purpose, standard derivatized glucosamine hydrochloride dissolved in HCl 0,1 N was analysed. As for amino acids, the derivatization reaction was essential to make glucosamine visible through chromatographic analysis. Being an amino sugar, in fact, glucosamine present an amino group that react well with the amino acid derivatization reagent (AccQ-Tag), as already reported by Díaz et al. 1996. From the UPLC-ESI/MS analysis, as already observed in other GlcN retention studies (Han & Heinonen, 2021; Sanches-Silva et al., 2012), it was possible to observe the separation of GlcN- AccQ Tag in the two anomers, alpha and beta, with retention times respectively of about 3.8 and about 7,3 min. The most intensive precursor ion of the glucosamine peaks was m/z 350.1 corresponding to the derivatization product between glucosamine and AccQ Tag. The glucosamine peaks were

found in the insect samples with the same characteristic masses (m/z) and the same retention times of the standard, together with the derivatized amino acids, and no interferences were observed with the peaks of amino acids-AccQ Tag, ensuring the applicability of the method for the simultaneous determination of GlcN and total amino acids derivatives in insects (Figure 3.1).

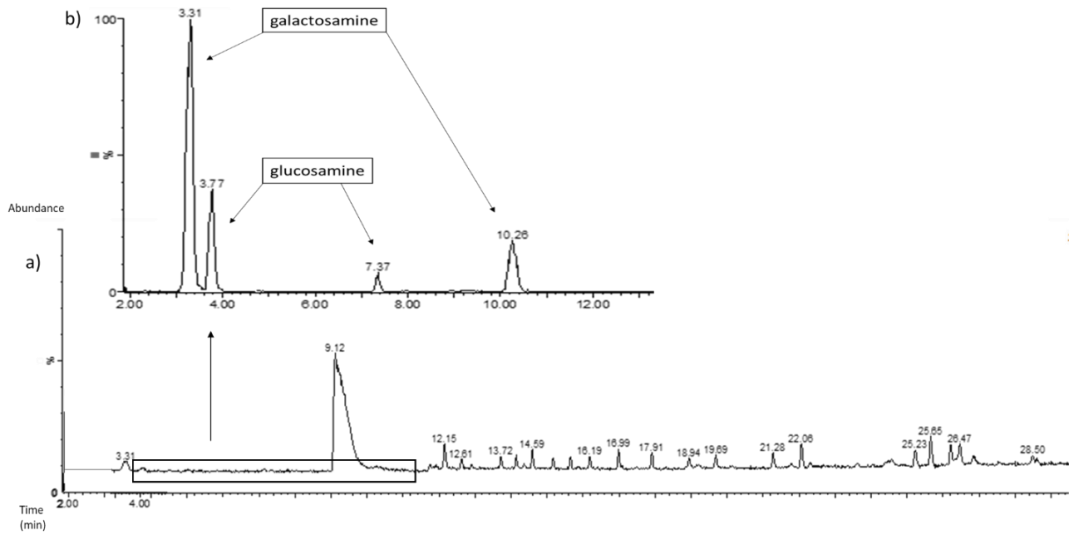

Figure 3.1 (a) total chromatogram of Glucosamine and Aminoacids of a Black soldier fly prepupa sample, (b) eXtracted ion chromatogram (XIC) of glucosamine, and galactosamine (internal standard) (m/z 350.1).

3.3.1.2 Choice of the internal standard

While 5 mM Norleucine in HCl 0,1 already selected by the method described by Caligiani A., et al. (Caligiani et al., 2018) was maintained as internal standard for the amino acid determination, it was necessary to choose an internal standard for the quantitative analysis of glucosamine. In literature, L-cysteic acid, L-norleucine, D-galactosamine and α-aminobutyric acid are the molecules most widely used as internal standard in the chromatographic methods for the analysis of glucosamine (Díaz et al., 1996; Flannery et al., 2001; Geraedts et al., 2011; Hagen, 1993; Liu et al., 2013). However, to obtain more precise and reliable measurement of chitin in the sample, an internal standard as close as possible to the analyte should be chosen and added in a similar concentration. Due to its easily

availability on the market and very similar molecular structure to glucosamine, the amino sugar galactosamine was chosen as internal standard. Galactosamine was added after hydrolysis and before derivatization protocol, generating two anomeric signals not interfering with the glucosamine peaks (retention time respectively of about 3,3 and about 10,4 min, fig 1 (b).

3.3.2 Method validation

The UPLC-ESI/MS method for the simultaneous quantification of total amino acids and glucosamine was subjected to validation in terms of linearity, detection and quantification limits, precision, and accuracy following the recommendations of the International Conference on Harmonization – Validation of analytical procedures: Text and Methodology (ICH (2005).

3.3.2.1 Linearity

Three potential external reference standards (glucosamine, N-acetyl glucosamine, and chitin) were selected for chitin quantification, as reported in paragraph 3.2.3. Linearity was tested for glucosamine hydrochloride, glucosamine hydrochloride hydrolysed in the same conditions of the method, hydrolysed N-acetylglucosamine and hydrolysed chitin, in the concentration range 0,25-1,5 mM, expressed as glucosamine concentration. Every stock standard solution was prepared in duplicate. The linear regression equations are reported in table 3.1, demonstrating a good linearity for all the tested standards, except for hydrolysed glucosamine, supposedly for the fastest and most unpredictable degradation of the free monomer in so strong hydrolysis conditions

Table 3.1 Linear regression equations and R^2 of the different standard tested and the purified standard chitin values (%) respectively recovered using each standard curve.

Standard	**Regression equation**	**R^2**	**Recovery Chitin determination in purified chitin (%) using the corresponding standard calibration curve**
Hydrolysed Practical Grade Chitin	y = 0, 5316x + 2,00556E-05	0,9946	103 ± 4
GlcN	y= 1,1836x-0,0019	0,9922	61 ± 3
Hydrolysed GlcN	y= 0,7821x-0,0003	0,9382	87 ± 5
Hydrolysed GlcNAc	y= 0,7811x-0,0014	0,9938	91 ± 5

In order to select the one allowing the most accurate quantification of chitin in insect samples, each calibration curve was tested for the quantification of a purified chitin sample (purity of 101,5% ± 1,5%). Results are reported in table 3.1. By observing the recovery results, it was found that only using the calibration line built with hydrolysed practical grade chitin as external standard, the recovery of the control sample was total (103 ± 4 %). This result could be easily explained by the fact that the two samples, being both polymers, degrade during hydrolysis in a similar way, allowing a more accurate measurement of the analyte. A lower recovery value of the control sample was instead found using the other curves, built by using as reference standard the non-hydrolysed glucosamine, hydrolysed glucosamine, and hydrolysed N-acetyl-glucosamine. The lowest recovery was found to be 61 ± 3 %, when using for calibration GlcN non subjected to hydrolysis. As explained, with this method chitin content was determined as amount of glucosamine released from chitin

fraction of the samples after strong acid hydrolysis. The conditions of this step may affect the release of glucosamine from insect chitin, also causing its partial degradation. For this reason, even if in general GlcN is one of the main external references used to quantify chitin, its use can expose to an incorrect quantification, and in particular an underestimation of chitin content of the insect sample due to a possible glucosamine degradation during acid treatment of the sample only. A higher, but not optimal recovery percentage of the chitin control sample, respectively of 87± 5 % and 91 ± 5%, was found by using the acid-treated standard monomers of GlcN and GlcN-Ac. This was probably due to a different behaviour of the standards (monomers) respect to chitin during acid hydrolysis. In fact, as reported by D'Hondt (D'Hondt et al., 2020), free monomers glucosamine and N-acetyl glucosamine, compared to whole biopolymer chitin, under the same condition of acid hydrolysis (extended periods and high temperature) have a different behaviour. The chitin polymer in a biological matrix could have a gradual, and supposedly incomplete release of glucosamine, which degrades differently respect to the pure glucosamine standard under the same hydrolysis conditions, leading to a final incorrect estimation of the sample chitin fraction. Consequently, the only way to obtain accurate chitin quantification is by using as reference standard a polysaccharide which during hydrolysis has an analogous behaviour respect to the chitin present in the sample.

The commercial practical grade chitin subjected to hydrolysis was therefore used as standard to evaluate the limit of detection and quantification, the precision, and the recovery of the analytical procedure and to quantify chitin content in BSF prepupae. This obviously implies that all the quantitative results are obtained in terms of chitin content, being chitin the external standards, even I f the actual analyte detected is glucosamine.

The linearity of the method was also tested in matrix, using an insect sample (BSF prepupae) spiked with different amount of practical grade chitin, as reported in 2.7.1. The spiked samples were then subjected to hydrolysis. The experiment was carried out in triplicate. The linear regression equation obtained for the calibration curve in matrix was y= 0,3605x + 0,014 with a correlation coefficient > 0,96, demonstrating the linearity of the methods also in matrix. Calibration curve in matrix was also used to calculate the real

starting concentration of chitin in the prepupa sample and, by comparing its slope with that one of external calibration line, to evaluate the presence of a matrix effect. These aspects are deeply described in paragraph 3.3.2.2.

3.3.2.2 Evaluation of hydrolysis condition

The main novelty and aim of this work were to quantify total chitin in insects together with total amino acids. The release of amino acids from protein requires strong acid (HCl 6N), long times (23h) and high temperature (110°C). In these conditions also chitin is hydrolysed releasing glucosamine that can be analysed in UPLC-ESI-MS simultaneously with the amino acids. However, as reported by D'Hondt et al. (D'Hondt et al., 2020), for the determination of chitin content is recommended a hydrolysis time not more than 10 hours to avoid a monomer degradation during the procedure. More precisely 4-6 h and 6-8 h were the optimal hydrolysis times respectively for the whole insect and for commercial chitin. These mentioned hydrolysis times are clearly less harsh than those used in this work. Therefore, to be sure that the long hydrolysis time here applied for amino acid release were also suitable for obtaining glucosamine from chitin, a BSF sample, analysed in triplicate, was hydrolysed for 6h at 110°C in 6 mL of HCl 6N. Also, practical grade chitin reference standard was hydrolysed in the same conditions of the samples and used to prepare the five working solutions to build the external calibration and quantify chitin amount. Data obtained show that the content of chitin determined after 6h (2,4 ± 0,1 %) and 23h (2,9 ± 0,5 %) of hydrolysis was not significantly different when the calibration is made by treating the chitin standard in the same conditions of the sample. These preliminary data suggested that the method normally used to release amino acids from protein is also suitable for chitin hydrolysis. Based on these encouraging results the method was subjected to optimization and validation, as reported in the following paragraphs.

3.3.2.3 Recovery test

Due to the absence of a "blank" insect sample a recovery test was carried out in matrix on BSF prepupa samples. To obtain the initial analyte concentration in the sample an internal calibration line was built by adding to grinded BSF prepupa samples different amounts of

Practical Grade standard chitin to obtain five levels of concentration of glucosamine, as described in paragraph 3.2.6.1. and it was compared with an external calibration line of the same standard chitin (Figure 3.2).

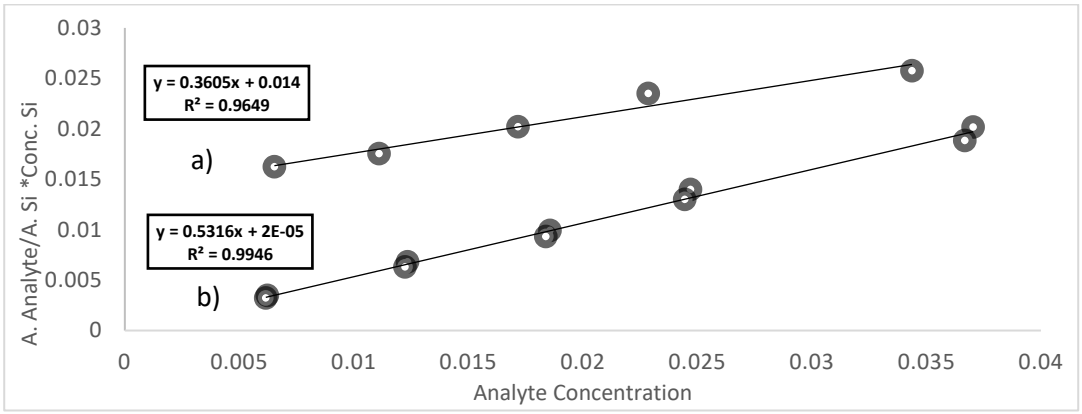

Figure 3.2 Calibration curves with linear regression equations and correlation coefficient (R^2) generated by plotting peak areas versus chitin concentration (0.25-1.5 mM, expressed as glucosamine concentration after hydrolysis) of a) practical grade chitin reference standard in BSF prepupa matrix; b) practical grade chitin reference standard, A: Area, SI: internal Standard, CONC: concentration

Utilizing the equation of the internal calibration, the real glucosamine concentration of the sample was obtained at y=0, graphically represented by the concentration at the intercept of the calibration line with the x axis. This value was found to be 0,97 mM in the sample being analyzed, corresponding to 9,8 ± 0,3% of chitin on insect dry mass. This result can be considered in line with chitin values found by the validating method in the no spiked prepupae and with the data reported in literature regarding the average chitin content in BSF prepupae (Caligiani et al., 2018; D'Hondt et al., 2020; Soetemans et al., 2020) where it always remains around 8-10% on DM.

Once found this starting concentration value, the recovery of chitin was performed by using the spiked insect samples mentioned above. After adding the different Practical Grade chitin weights to the samples, they were subjected to hydrolysis and derivatization protocol and the monomer recovery was determined by using UPLC-ESI/MS. Results are reported in table 3.2.

Table 3.2. Recovey of GlcN by UPLC-ESI/MS method after acid hydrolysis of chitin with 6 M HCL for 23 h under 110°C. * % derived from the real chitin starting amount calculated with the in matrix line plus the chitin amount added ** calculated chitin percentage vs expected

Amount of commercial practical grade Chitin spiked (mg)	Amount of GlcN Expected in the sample (mg) ± SD *	Amount of GlcN calculated in the sample (mg) ± SD	Recovery (%) ± SD **
26,8	44,9 ± 0,3	32 ± 4	71 ± 9
17,9	35,7 ± 0,8	29 ± 1	82 ± 5
13,4	31,2 ± 0,5	25 ± 3	81 ± 9
8,9	26,4 ± 0,6	21,9 ± 0,5	83 ± 4
4,4	22,7 ± 0,2	21 ± 3	90 ± 15

As it is possible to observe from figure 3.2 and from the recovery results (table 3.2), the higher is the amount of chitin in the sample, the lower the recovery of the monomer. According to the validation criteria (DIR 2001/22/CE), the recovery should remain between 80 and 100%, and the corresponding concentrations range allowing an acceptable chitin determination goes from 0,25 to 1 mM, expressed as glucosamine. This is probably related to a matrix effect, evidenced also from the comparison of both calibrations, internal and external. In fact, the angular coefficients of the two calibration curves are different, suggesting the need to analyse samples in the specific range allowing more than 80% of recovery. Alternatively, it could be necessary to make for each analysis an internal calibration.

3.3.2.4 LOD and LOQ

The instrumental Limit of Detection and limit of Quantification were calculated utilizing the S/N ratio methods, based on the determination of the peak-to-peak noise (Apostol et al., 2009). LOD and LOQ were therefore calculated as the concentrations of practical grade chitin producing after hydrolysis a recognizable glucosamine peak with a S/N ratio of,

respectively, 3,3 and 10. At least, with the conditions of this method the limit of detection (S/N ratio of 3,3) and the limit of quantification (S/N ratio of 10) values corresponded respectively to a concentration of 0.00068 mM and 0,00204 mM of chitin, corresponding, according to the present method, to a final concentration in a solid sample of 0.04 %, and 0.11% respectively.

3.3.2.5 Repeatability

Repeatability was calculated at first as intraday precision (intermediate precision) on a BSF prepupae sample spiked with five chitin concentration levels (0.25, 0.5, 0.75, 1, 1.5 mM expressed as glucosamine concentration).

Samples were prepared using the same procedure (described in paragraph 3.2.7.1) and equipment, in the same laboratory and were analysed in triplicate within one day. The results of precision, expressed in table 3.3, were compared with Horwitz predicted intra-laboratory precision (PRSD), calculated as $0,66 \times 2 \times c - 0.1505$, where c is the concentration level expressed as a mass fraction. A HORRAT value of less than 2 commonly indicates satisfactory precision results. From the repeatability results obtained (table 3.3) is possible to observe that the Horwitz equation is always satisfied, also in the presence of different concentrations of chitin. It is therefore possible to affirm that the method has a good intraday precision in the range of concentration considered.

Table 3.3 intra-Day Repeatability of the Method, calculated on BSF prepupa spiked with different chitin levels.

Chitin level (mM expressed as glucosamine)	Intraday repeatability (RSD %)	Horwitz ratio for intraday repeatability
0,25	3.02	0,9
0,5	1.54	0,4
0,75	0.53	0,1
1	3.12	1,0
1,5	2.87	0,9

Due to the possible lability of the GlcN and derivatized GlcN monomer, it was also interesting evaluate whether the precision of the method was maintained over time and when it could no longer be defined as acceptable. Therefore, repeatability was also calculated as inter-day precision on a period of four weeks by analysing once a week a sample of hydrolysed practical grade chitin and a sample of hydrolysed BSF prepupae, prepared using the same procedure, in the same laboratory. One set of hydrolysed practical grade chitin and BSF prepupae was stored at -20°C (acid environment), executing the derivatization ex novo every week, just before the analysis. A second identical set of hydrolysed practical grade chitin and BSF prepupae was instead derivatized with the AccQ Tag and stored at -20°C for the following analyses. The quantification of GlcN of each set of samples was done by preparing every week an independent and fresh practical grade chitin calibration line. The results, reported in table 3.4, were compared with Horwitz predicted intra-laboratory precision (PRSD).

Table 3.4. Inter day Repeatability of the method, calculated on Practical Grade Chitin and BSF Prepupa in two different storage conditions.

WEEK	Practical Grade Chitin (Stored after derivatization)		Practical Grade Chitin (Derivatized immediately before the analysis)	
	Recovery (%)	RSD%	Recovery (%)	RDS%
1	109 ± 5	30.9	97 ± 4	32.2
2	63 ± 2		65 ± 2,2	
3	58 ± 1		55 ± 1,5	
4	50 ± 2		49 ± 1,3	
	BSF Prepupae (Stored after derivatization)		BSF Prepupae (Derivatized immediately before the analysis)	
	Quantification (% on WW)	RSD%	Quantification (% on WW)	RSD%
1	2,7 ± 0,2	24.7	2,7 ± 0,2	21.6
2	2,7 ± 0,5		2,2 ± 0,5	
3	1,8 ± 0,4		1,8 ± 0,3	
4	1,7 ± 0,3		1,7 ± 0,3	

The first evidence shown from the results is that the recovery and the chitin quantification followed the same trend during the time, whether the GlcN was derivatized at the moment or remained derivatized throughout the entire course of the experiment (four weeks). This result suggests that the derivatization step is not the main factor affecting the stability of the analyte. On the other side, results suggest that the stability of GlcN overtime in the hydrolysed practical grade standard of chitin is different from the stability of the monomer in the real hydrolysed prepupae sample. In fact, comparing the two samples (table 3.4) it can be noted that the GlcN degradation in the standard chitin is faster than in the real prepupa sample. In the insect sample the recovery of the analyte remains identical in the

first two weeks of the experiment, while for the standard chitin, already after the first week, it drops to a recovery around 60%. This result suggests that the stability over time of the monomer is related to the matrix. As reported by D'Hondt et al. (2020), in-fact, the GlcN behaviour mostly depend on the sample characteristics, such as its chitin content or, in the case of a biological sample, the presence of other molecules potentially affecting the analyte protection. In this regard, the absence of other substances in the commercial standard, probably lead to a faster degradation of the free analyte during the storage. Instead, the lower amount of chitin and the presence of other components in the insect body bring to a reduced degradation during the time of the analyte. Hence, the less quantity of glucosamine released, and it is bound with the other compounds probably protect it during a long-term storage from an excessive degradation.

Finally, the precision parameter, evaluated through l'RSD% calculated on the average of the quantification and recovery values found during the time in the two different matrices, suggests that in any case the analysis should be performed immediately after the samples preparation to obtain more precise results and prepare always a fresh hydrolysed practical grade chitin standard solution to build the external calibration, avoiding possible errors due to the matrix and time of storage.

3.4. Method application

3.4.1 Amino acids and chitin content in insects and food supplements.

After optimization and validation, the quantitative method developed was applied for the simultaneous determination of total amino-acids and chitin in insect samples and then extended to other food matrices containing both polymers of glucosamine and proteins. Specifically, two insect species (*Hermetia illucens* and *Alphitobius diaperinus*) were considered, based on their large use in the developing edible insect industry. The results related to amino acids and chitin content in the two insect samples, obtained on three replicates and reported as average percentage ± DS on dry weight, are showed in table 3.5.

Table 3.5. total amino acid and GlcN (as equivalent chitin) content of Black soldier Fly prepupae (expressed as g/100 DM) compared with other matrices contented AA and chitin (*Alphitobius diaperinus* larvae, supplement 1 and supplement 2). The results are the mean of triplicate analysis. *expressed as chitin equivalents

	Chitin (%DW)	Total AA (%DW)
Hermetia illucens prepupae	9,4 ± 0,8	43,9 ± 0,7
Alphitobius diaperinus larvae	7 ± 1	53 ± 1
Supplement 1	13 ± 2*	15 ± 1
Supplement 2	5,3 ± 0,1*	87 ± 3

The amino acids content found with our method fall perfectly in the range of 37-56% from the literature (Bosch et al., 2014; Sánchez-Muros et al., 2014; Spranghers et al., 2017). Regarding chitin content, the new method reports an average chitin amount in BSF prepupae of 9,4% ± 0,8 dry matter basis. If compared with the chitin amount obtained from indirect chitin quantification methods, this value is quite different. For example, if theoretically calculated as reported Caligiani et al (2018) i.e., by difference from the protein content, the chitin content in BSF prepupa is found higher than ours, ranging from 11.7% and 14,6%. If calculated following the method of Liu et al., 2012 (based on the weight of the residue obtained after the total elimination of protein and mineral) the chitin amount calculated is lower, around 6-7% (Spranghers et al., 2017). These differences are not so evident when the chitin percentage obtained with our simultaneous method is compared to the value obtained using direct quantification method. The chitin content in our samples results in fact in accord with the result of Caligiani et al. (2018) and D'Hondt et al. (2020) which used for the analyte quantification respectively GC-MS and LC analysis. Regarding the in Alphitobius diaperinus larva, as a whole the AA and chitin amount obtained from the simultaneous method, are coincident with those reported in literature. The chitin value only results slightly higher respect the results found in previous works. In the works of Leni et al. (2019) and Han and Heinonen (2021) (Han & Heinonen, 2021; Leni, Caligiani, et al., 2019), for example, the chitin percentage is lower (around 4,5%) as compared to ours. Despite all, the discordances found are negligible considering that the differences in diets,

rearing conditions, larval stage etc. can affect the compositional properties of the same insect species (van Huis et al., 2013). With this new method is therefore possible to avoid the errors which occurs when indirect methods are used and to obtain the same accurate results of direct quantification methods, but analysing two main insects components simultaneously, dramatically reducing the analysis times. In order to extend the applicability and evaluate the efficiency of this developed quantitative method also on others matrix, two different commercial supplements containing glucosamine in the form of glucosamine hydrochloride or sulphate, chondroitin sulphate (supplement 1 and supplement 2) together with a protein source were analysed. The samples were prepared using the same conditions applied for insect samples. However, because the external calibration curve for the quantification of chitin, is built using practical grade standard chitin, the results obtained are directly expressed as chitin amount. Hence, analysing samples containing free glucosamine or other glucosamine-containing polymers and not chitin, it is necessary to take in consideration that the final values of glucosamine, using this calibration, will be expressed as chitin equivalents. The effectiveness of the method also on these commercial matrices could result very attractive for the pharmaceutical field, making possible to quantify faster and precisely amino acids and glucosamine and assess the veracity of what is indicated on the label. The results obtained from the two samples analysed, showed in table 3.5, were therefore compared with the values declared by the company on the label. According to the label, the supplement 2 is almost totally composed by AA, while glucosamine amount is present in a very little percentage (around 3%). Regarding supplement 1, it was labelled as having 15% of glucosamine and 12% of proteins. Despite the limitation of using chitin as external calibration, after the comparison of our results with the labels it was possible to confirm the agreement between the values found by UPLC-ESI/MS and what declared on label by the productors, confirming the efficiency of the method also on non-biological matrices.

3.5 Conclusions

In this work a UPLC-ESI/MS method already used for the total amino acid determination in insect matrix was optimized and validated also for the simultaneous quantification of chitin. The acid hydrolysis carried out on the insect sample indeed allows not only the release of amino acids from the protein fraction but also of the glucosamine from chitin. The method was thus adjusted in order to detect also free glucosamine after hydrolysis, from which is possible to derive the insect chitin content. This newly developed UPLC-ESI/MS method proved to be simple and fast, and no fundamental modifications were needed as compared to the analysis of amino acids. During the validation tests the method showed a good linearity, precision, recovery, and limits of quantifications for chitin. Moreover, from the results obtained from the analysis a precise and accurate determination of insect protein and chitin fraction was obtained. Moreover, the efficiency of the present UPLC-ESI/MS method allows its application not only to the insect matrices, but also to other biological substrates or all those commercial preparations containing chitin, glucosamine or other polysaccharides holding N-acetyl-glucosamine (for example: hyaluronic acid) and proteins.

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CHAPTER 4

Fermentation by Lactobacillus spp. Modifies the molecular composition of black soldier fly prepupae and-derived biomasses

Part of the content of this chapter is based on the following publication:

Hadj Saadoun, J., Luparelli, A. V., Caligiani, A., Macavei, L. I., Maistrello, L., Neviani, E., Galaverna, G., Sforza, S., & Lazzi, C. (2020). Antimicrobial biomasses from lactic acid fermentation of black soldier fly prepupae and related by-products. *Microorganisms*, *8*(11), 1785. https://doi.org/10.3390/microorganisms8111785

It is also under drafting for the submission as:

Anna Valentina Luparelli, Jasmine Hadj Saadoun, Camilla Lazzi, Stefano Sforza, Augusta Caligiani, Fermentation by *Lactobacillus* spp. Modifies the molecular composition of black soldier fly prepupae and-derived biomasses.

Abstract

Solid state fermentation is a process often applied to valorise organic wastes, thanks to the countless benefits this process can bring to the unexploited biomasses, such as the microbial stabilization and the simultaneous production of antibiotics and bioactive compounds. Hermetia illucens (Black Soldier Fly, BSF), a non-pathogenic insect belonging to the Stratyiomidae family, is as well becoming a popular means of biowaste's valorisation through bio-conversion processes of residual biomasses into biofertilizers, biodiesel, feed ingredients etc. In a contest in which the interest in waste materials management and enrichment is growing considerably, it was wanted to find a way to valorise also the BSF derived wastes generated during the development of the insects on agri-food residues and based on their resulting composition, find them useful applications. In this work, for the first time, a solid-state fermentation was conducted with 2 lactic acid bacteria (LAB) strains, isolated from food, on BSF extra prepupae biomass and BSF-derived wastes (pupal membranes and adult insects at the end-of-life cycle) produced during bioconversion processes. The present work was undertaken to study the difference in the molecular composition between fermented and unfermented insect-derived waste materials, focusing on lipid, protein, and chitin fraction. from the bulk evaluation of fat, protein, moisture, and ashes composition of insect-derived biomasses was carried out using standard procedures (AOAC, 2002). Fatty acid profiles were determined by GC-MS, whereas total amino acid analysis, glucosamine monomer content (after chitin hydrolysis) and the formation of possible peptides or chitin oligomers was carried out by LC/ESI-MS methodologies. A metabolomics approach by 1H NMR was also applied to get further insight in lipid insect fraction. The results showed that the lipid fraction is the most affected by the fermentation: the fermented biomass had a more complex fatty acid profile as compared to unfermented mass, with a fatty acid composition shifting from a typical one from BSF to a more typical one for LAB. Also, the protein fraction changes upon fermentation, especially in the amino acid composition and in the production of small peptides. On the contrary no chitin oligomers formation was observed after the fermentation process. This study shows how fermentation can modify the molecular

composition of insect-derived waste, exploiting the typical metabolic behaviour of fermenting bacteria. Further studied are encouraged to better understand possible functional properties of insect fermented biomasses and related applications.

4.1 Introduction

The current constant increase in world population results in the continuous expansion of the agriculture sector. Beside the increased soil consumption, the number of organic by-products generated by agriculture industries increases every year and adequate disposal procedures are required to avoid the negative effects of their accumulation on the environment and animal health (Sadh, Duhan, et al., 2018). The organic leftovers are indeed rich in potential due to their still high nutritive value (Barbi et al., 2020; Graminha et al., 2008; Rodriguez Couto, 2008). In the last decades, several studies reported that one possible strategy to add value to wastes from organic compounds is solid state fermentation (SSF) (Hadj Saadoun et al., 2021; Pandey et al., 2000; Sadh, Duhan, et al., 2018; Wang & Shelomi, 2017). From a nutritional point of view, optimal and enhanced composition of fermented food was shown to improve human health, prevent diseases, and enhance food safety (C. Borresen et al., 2012; Chelule, P. K., Mokoena, M. P., & Gqaleni, 2010; Hasan et al., 2014). When applied to food wastes, SSF can also bring significant advantages, in terms of valorization and product recovery. As a natural result of their growth, in fact, the microorganisms involved in the process enrich the residual biomasses with their own metabolites, allowing the waste reuse in many industrial sectors (Thomas et al., 2013).

Among the bacterial species, Lactic Acid Bacteria (LAB), a heterogeneous group with status recognition as Qualified Presumption of Safety (QPS) by EFSA (Koutsoumanis et al., 2020), are the most used for organic biomass fermentation, and thus exploited for industrial bioprocesses. Their adaptation to specific substrates leads to the production of organic acids, volatile fatty acids, antibiotics, bioactive peptides, etc. which improve the nutritional value, digestibility, and potentialities of organic substrates. The acid environment generates during the fermentation facilitates the LAB endogenous enzymes activity, which operates on the macronutrients (lipids, proteins, polysaccharides) of the starting food matrix, increasing bioavailability of proteins and simple sugars and generating as well other bioactive compounds with positive effects on the final value of the product. The organic acids produced, mainly lactic and acetic acids, contribute to the lowering of the pH and

consequently contrast the pathogens grow, prolonging the shelf life of the product. The ability of LAB to produce microbial and fungal inhibitory metabolites, including hydrogen peroxide and bacteriocins, brings tangible advantages, such as the possibility of using the fermented biomasses as natural antimicrobials. LAB fermentation is also known to facilitate the mineral intestinal absorption, increase the amount of the vitamins in the substrate, and detoxify mycotoxins (Chelule et al., 2010; Santos et al., 2008; Sybesma et al., 2003).

LAB fermentation can give an industrial-scale interest to an under-utilized biomass, supporting the circular economy strategy (Nout, 2009; Obiri-Danso et al., 1997; Sabater et al., 2020).

Insects have also been demonstrated to be important bioconversion tools to valorize agro-industrial by-products (Barbi et al., 2020; Salomone et al., 2017; Surendra et al., 2016). Insects grow and reproduce easily on many organic wastes, and numerous species are able to implement a bioconversion mechanism (with high conversion rate) on organic residue to obtain a biomass rich in particular of proteins and lipids (Jansson & Berggren, 2015; Dennis G.A.B. Oonincx & de Boer, 2012) (Cappellozza et al., 2019; Lohri et al., 2017). *Hermetia illucens*, also called black soldier flies (BSF), is one of the insect species most commonly involved in bioconversion of underutilized by products into higher nutritional biomasses. BSF can be used as feed ingredients thanks to the optimal nutritional composition particularly abundant in lipids and essential ammino acids and very similar to several feed preparations (Alagappan et al., 2021; Čičková et al., 2015; De Smet et al., 2018; Erickson, M. C., Islam, M., Sheppard, C., Liao, J., & Doyle, 2004; Surendra et al., 2016). Processed proteins from farmed edible insects, including BSF, were already permitted by the European Commission for feeding aquaculture animals as molluscs, crustaceans, and fishes (Reg. 2017/893/UE). Nowadays, insects are also allowed for feeding chickens and pigs, as of 17 august 2021, Commission Regulation (UE) 2021/1372 was enacted. BSF larvae are also already reared for pet feed (Wang & Shelomi, 2017). Moreover, the amendment of January 2018 of the novel food regulation (2015/2283) has led to the inclusion of whole insects and their parts in the list of novel foods. From this moment more than twenty applications were submitted to the European Food Safety Authority (EFSA) for insects to

be assessed as novel food. BSF is considered part of the eleven insects species with the greatest potential to be used also as food (European Council Regulations No. 2015/2283, 2015).

As a result of the above potential applications, there has been an increase in BSF rearing on food by-products on a large scale in the last years. BSF food-waste bioconversion ability is the object of a lot of studies in literature, both as a waste control tool and for the enriched nutrient profile of the resulting biomass (Manzano-Agugliaro et al., 2012; Rumpold & Schlüter, 2013; Van Huis, 2013). On the contrary, there is a lack of information related to the potential environmental impact that a large-scale insect-rearing process can cause. These data are particularly important to compare the benefits of intensive insect production on organic wastes with other alternative sources as solutions for food waste management and the rising global demand for food and feed. As reported by Salomone at al., 2017, approximately from 10 tonnes of food-waste can be produced 300 kg of dried larvae and 3,346 kg of compost (frass) (Salomone et al., 2017). Also, in an industrial mass rearing of BSF, besides frass several other by-products are generated. Among these, there are the puparia, which are the shells left after the adult emergence, and the bodies of the adults after mating and egg laying. Moreover, the employment of an insect species such as *Hermetia illucens* on a large-scale breeding, due to the very high growth factor and the small life cycle, can generate an excess of prepupa biomass compared to the market demand, with no immediate applications. Studies are needed in order to better understand the molecular composition on insect by-products and related potentialities in food/feed market sectors. In a preliminary work, with the aim of supporting the circular economy, a LAB fermentation by using two different strains, *Lacticaseiobacillus rhamnosus (1473) and Lactiplantiobacillus plantarum (285)*, was applied as a method to valorize BSF prepupae and derived biomasses and evaluated their antimicrobial activity. Data related to microbial challenge tests performed are outlined below. In this previous work, also a preliminary molecular composition analysis of the BSF substrates after fermentation was assessed (Hadj Saadoun et al., 2020). For a deeper examination of this last aspect, in our work a detailed molecular composition of unfermented BSF prepupae and related by-products

derived from bioconversion processes was determined. As a further step, to evaluate potential improvements to the nutritional properties of the insect biomasses following a fermentation as described in the work cited above, the differences in molecular composition before and after fermentation were accurately explored.

4.2. Materials and methods

4.2.1 Prepupae and derived by-products samples

The BSF prepupae and derived biomasses (puparia and adult flies) were kindly provided by the Laboratory of Applied Entomology - BIOGEST-SITEIA, Department of Life Science, University of Modena and Reggio Emilia, Reggio Emilia (RE), Italy. The breeding conditions of BSF larvae until they reach the prepupae stage and the way BSF adults and puparia samples were collected are the same discussed by Hadj Saadoun et al. (2020)

4.2.2 Fermentation

Fermentation of BSF samples was carried out as reported by Hadj Saadoun et al. (2020). Briefly**,** *Lacticaseibacillus rhamnosus* 1473 isolated from Parmigiano Reggiano cheese, and *Lactiplantibacillus plantarum* 285, isolated from Brazilian cheese, were chosen for fermentation. Both the strains belonged to the collection of the Department of Food and Drug (University of Parma, Parma, Italy). The strains were maintained at -80°C in De Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) supplemented with 12.5% glycerol (v/v). Finely ground prepupae were combined with sugar (8.5% w/w), while BSF Puparia and adults' powders with sugar (8.5% w/w) and water (70% v/w). All the samples were then sterilized in autoclave at 121° for 20 minutes in a glass jar. Then, 30g of each BSF substrates were incubated with a final concentration of 7 Log CFU/g of each bacterial suspension for 72 h at 30 °C for *L. plantarum* 285 and at 37 °C for *L. rhamnosus* 1473. Each fermentation was tested in triplicate.

In this work both unfermented and fermented (with the two different LAB strains) BSF prepupae and derived biomasses were analysed. The sample's nomenclature is listed below: Unfermented Adult: AD_Unf; Adult fermented with *L. rhamnosus* 1473: AD_1473;

Adult fermented with *L. plantarum* 285: AD_285; unfermented Puparia: PUP_Unf; Puparia fermented with *L. rhamnosus* 1473: PUP_1473; Puparia fermented with *L. plantarum* 285: PUP_285; unfermented Prepupa: PRE_Unf; Prepupa fermented with *L. rhamnosus* 1473: PRE_1473; Prepupa fermented with *L. plantarum* 285: PRE_285.

4.2.3 Microbial challenge test

Challenge tests were carried out to evaluate the growth potential of *Listeria monocytogenes* LMG 21264, *Escherichia coli* K88, *Salmonella* RISSEN in fermented and unfermented samples, in triplicate. Before use, pathogenic strains were cultured twice, for 24 h at 37 °C, with a 3% v/v inoculum in TSB added with 0.6% yeast extract (Oxoid, Basingstoke, UK). Afterward, TSB broth was inoculated (3% v/ v) with each revitalized strain and incubated for 15 h at 37°C, to obtain a cell concentration of 9 Log CFU/mL. Each bacterial culture was then centrifuged (12,857x g, 10 min, 4 °C), washed twice in Ringer solution, and suspended in sterile bidistilled water. Puparia, adults, prepupae fermented with both strains and unfermented (control), were inoculated, individually, with each bacterial suspension of the pathogen, to obtain a final concentration of 6-7 Log CFU/g. The inoculated substrates were incubated at 37°C and analysed immediately after the inoculum (T_0), after 24 h (T_{24}), and after 48 h (T_{48}) of incubation. For microbial counts, 5 g of each sample were homogenized in 45 mL of Ringer solution for 60 s in a Stomacher, and serial 10-fold dilutions were performed. The total viable count was determined using plate count agar on selective medium for each pathogen: Microbiology Chromocult (Merck KGaA, Darmastadt, DE) for the detection of *Salmonella* Rissen and *Escherichia coli* and Listeria selective agar base acc. Ottaviani and Agosti (ALOA) (VWR, Leuven, BE) for *Listeria monocytogenes*.

4.2.4 Proximate composition

Samples were grinded for 2 minutes with IKA A10 laboratory grinder before analysis. Standard procedures (AOAC, 2002) were used to test moisture, lipid and ash composition of BSF biomasses before and after fermentation, Moisture was determined in the oven at 105 °C for 24 h. An automatized Soxhlet extractor (SER 148/3 VELP SCIENTIFICA, Usmate

Velate, Italy) was used to determine crude fat, extracted using diethyl ether as solvent. Total ash was determined after mineralization at 550 °C for a total time of 10 h (5 h + 5 h). Total protein and chitin content of the samples was calculated respectively from the sum of the amounts of amino acids and of free glucosamine released after acid hydrolysis, carried out as described in paragraph 4.2.5. To determine the correct protein and chitin amounts, the moles of each amino acid and of glucosamine determined were multiplied for their residual molecular mass (molecular mass of free amino acid subtracted from the molecular mass of water).

4.2.5 Proteins and chitin determination of insect samples

Protein and chitin content determination was simultaneously carried out by summing respectively the total amino acids and the glucosamine (GlcN) released from these two macro fractions after acid hydrolysis.

4.2.5.1 Total Amino Acids and chitin determination

Samples (0.5 g) were hydrolysed with 6 mL of HCl 6 N at 110 °C for 23 h. At the end of hydrolysis, 7.5 mL of 5 mM Norleucine in HCL 0.1 N, used as internal standard for total amino acid determination, was added. After a filtration in Buckner, the samples were brought up to a volume of 100 mL with deionized water. Therefore, 8 µl Galactosamine 46 mM, used as internal standard for glucosamine determination, were added to 450 µl of hydrolysate solution. The solution was adjusted to 500 µl with deionized water.

Only for the analysis of cysteine, determined as cysteic acid, the acid hydrolysis was preceded by performic acid oxidation, following the procedure described by Caligiani et al. (2018) (Caligiani et al., 2018). In this case, 2 mL of performic acid freshly prepared (by mixing formic acid with hydrogen peroxide in 9:1 proportion) was added to an amount of 0.5 g sample and kept in an ice bath for 16 h at 0 °C. Then the bromine formed after the addition of 0.3 mL of hydrobromidric acid was removed under nitrogen flow. The hydrolysed samples were analysed by UPLC/ESI-MS according to the technical specifications described in the following paragraph (4.2.5.2), after derivatization with reconstituted AccQ Tag reagent (Waters Co., Milford, U.S.A.) according to the method

described by Leni et al. (2020) (Leni et al., 2020). Calibration for total amino acids was performed with a standard solution prepared mixing 133.3 µl of Norleucine (5 mM), 133.3 µl of amino acids hydrolysate standard mixture (2.5 mM), 133.3 µl of cysteic acid in HCL 0.1 N (2.5mM), and 100 µl of deionized water. For chitin determination, calibration was performed by using five different concentrations, ranging from 1.5 mM to 0,1 mM of a standard solution prepared by mixing a commercial chitin (external standard) subjected to hydrolysis with the selected internal standard (galactosamine), both at 2,5 mM as final concentration.

4.2.5.2 Tryptophan determination by UPLC/ESI-MS after alkaline hydrolysis

Tryptophan analysis was carried out according to the method described by Caligiani et al. (2018) with some modifications. 3 mL of 4 N NaOH and 150 µl of 5-methyl- DL-tryptophan standard solution (prepared by mixing 16 milligrams in 100 mL of distilled water) were added to 0.2 hundred milligrams of sample and hydrolysed at 110 °C for 18 hours. After alkaline hydrolysis, the solution was neutralized by adding 37 % HCl and brought to 25 mL with sodium borate buffer (0.1 M, pH 9.0). The samples were centrifuged for 5 minutes at 4000 rpm at 4°C. 0.45 µm nylon filter membrane was used to filter the supernatants collected after centrifugation. UPLC/ESI-MS analysis was performed by using an ACQUITY UPLC separation system with an Acquity BEH C18 column (1.7 µm, 2.1x150 mm). The mobile phase was composed by H_2O + 0.2 % CH_3CN + 0.1 % HCOOH (eluent A) and CH_3CN + 0.1 % HCOOH (eluent B). Gradient elution was performed: isocratic 100 % A for 1.8 min, from 100 % A to 50 % A by a linear gradient in 11.4 min and 0.8 min at 50 % A plus washing step at 0 % A (100 % B) and reconditioning. The flow rate was set at 0.25 mL/min, injection volume 2 µl, column temperature 35 °C and sample temperature 23 °C. Detection was performed by using Waters SQ mass spectrometer: ESI source in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 150 °C, desolvation temperature 300 °C, cone gas flow (N_2): 100 l/h, desolvation gas flow (N_2): 650 l/h, full scan acquisition (100-2000 m/z), scan duration 1s.

4.2.6 Free amino acids profile determination

In addition to the total amino acid analysis, free amino acids profile was evaluated following the procedure described by Leni et al., (2020). 0.5 g of frozen and grinded BSF puparia, adults and prepupae, unfermented and fermented were weighted and homogenised under stirring with 5 mL of water and 0.34 mL of Norleucine 5 mM in HCl 0,1 as internal standard for two hours. The samples were brought to 10 mL with deionized water as final volume and transferred into 15 mL falcon tubes. Finally, they were centrifuged for 30 minutes at 4000 rpm at 4°C. The supernatants were collected, filtered using a 0.45 µm nylon filter membrane, and analysed by UPLC/ESI-MS after derivatization following the conditions described in points 2.4.1 and 2.4.2. Quantification was performed against a set of standard solutions.

4.2.7 Determination of the degree of Hydrolysis

The percentage of peptide bonds cleaved with respect to the total number of peptide bonds is defined as degree of hydrolysis (DH). The protein DH was calculated using o-phthaldialdehyde (OPA) method described by Spellman et al. 2003, with some modification (Spellman et al., 2003). It exploits the formation of an isoindole spectroscopically quantifiable at 340 nm. 100 mL of OPA/NAC (N-acetylcysteine) reagent was prepared by combining 10 mL of 50 mM OPA (in methanol) and 10 mL of NAC 50 mM (in methanol), 5 mL of 20% (w/v) SDS, and 75 mL of borate buffer (0.1 M, pH 9.5). The reagent was kept in dark and stirred for at least 1 h before use. The OPA assay was carried out by the addition of 20 µL of sample (or standard) to 2.4 mL of OPA/NAC reagent in a 5 mL of a plastic Eppendorf. Before the analysis, the samples were centrifuged at room temperature for 10 minutes at 1000 rpm. The absorbance of this solution was measured at 340 nm with JASCO B-530 UV–Vis-spectrophotometer (JASCO, Oklaoma City, OK, U.S.A) against a control cell containing the reagent plus 20 µL of borate buffer. The intrinsic absorbance of the sample was measured before OPA addition and subtracted. A standard curve was prepared using isoleucine (0–2 mg mL−1). The degree of hydrolysis (DH) was calculated as the ration between the free nitrogen groups after hydrolysis and the total nitrogen groups: DH%= (N free /N total) ×100. The first value was calculated by the OPA reactivity, and the total moles

of nitrogen atoms present before hydrolysis were calculated by the total grams of proteins divided by the average of residual amino acids molecular mass (Mw 110). The total grams of proteins were calculated from total amino acids analysis (paragraph 4.2.5.1).

4.2.8 Determination of the integrity of the chitin fraction after fermentation by UPLC-ESI/MS

A deeper study of the integrity of chitin fraction related to the possible formation of chitin oligosaccharides in BSF insect samples from fermentation process was carried out following an extraction procedure based on the protocol described by (S. Kim et al., 2003), with some modifications. Briefly, 10 mL of 10% aqueous solution of ethanol was added to 1 gr of BSF samples. The mixture was stirred at 50°C for 1h and centrifuged at 3900 rpm for 30 min at 20°C. Finally, 1mL of the supernatant was collected, evaporated under nitrogen, and redissolved in a solution of distilled water/acetonitrile 1:1. 400 µl were sampled for the analysis of the potentially extracted oligosaccharides from insect material. Chitin oligomers content in the extracts were checked by using an UPLC/ESI-MS analysis (ACQUITY UPLC® BEH Amide column (2.1 x 100 mm, 1.7µm). The mobile phase was composed by 80% CH3CN + 20% H_2O + 0.1% NH_4OH (eluent A) and 30% CH3CN + 70% H_2O + 0.1% NH4OH (eluent B). Gradient elution was performed: from 100% A to 40% A and 60% B by linear gradient in the first 10 minutes, from 40% to 100% A in 0.02 minutes, isocratic 100% A from 10.02 to 30 minutes. Flow rate was set at 0.17 mL/min, injection volume 2µL, strong needle wash 20% CH_3CN + 80% H_2O, weak needle wash 75% CH_3CN + 25% H_2O, seal wash 50% CH3CN + 50% H_2O, column temperature 35°C and sample temperature 18°C. Detection was performed by using Waters SQ mass spectrometer: ESI source in negative ionization mode, capillary voltage 2.8kV, cone voltage 25V, source temperature 120°C, desolvation temperature 350°C, cone gas flow (N_2) 50 L/hr, desolvation gas flow (N_2) 500 L/hr, full scan acquisition (100-2000 m/z).

4.2.9 Determination of fatty acids profile by GC-MS

The determination of fatty acids profile was carried out on the crude fat extracted using the Soxhlet extraction technique (Section 2.3). Before the analysis in GC-MS, acidic-

catalysed transmethylations was carried out on 50 mg of BSF fat residue according to the method used by Caligiani et al., 2018, with some modifications. Weighed fat of each BSF sample was dissolved in 1 mL of 5 % HCl in methanol. The reaction was carried out in oven at 70 °C for 45 minutes. After cooling, 50 µl of methyl tetracosanoate, used as internal standard, and 2.5 mL of hexane were added. The superior hexane phase containing the fatty acid methyl esters was collected and stored. Before the instrumental analysis, a dilution of each extract was performed by adding a different quantity of hexane to match the linearity range of the GC-MS instrument. The solutions were split injected (1µL) on a Thermo Scientific Trace 1300 gas-chromatograph (Thermo Scientific, Waltham, Massachusetts, USA) carrying a Supelcowax ms capillary column (30m, i.d 25 mm, Supelco, Bellafonte, USA) coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). The carrier gas was helium (1 mL/min), injector and detector temperatures were kept at 250 °C, while oven temperature was programmed from 80 to 240 °C at 20 °C/min. The content of every single fatty acid was calculated concerning the concentration of the internal standard, after calculating the response factors using the Supelco® 37 Component FAME Mix (Sigma Aldrich, Saint Louis, MO, USA). Finally, results were expressed as a relative percentage of each fatty acid.

4.2.10 1H NMR lipidomics

Distribution of the lipid classes was performed according to Caligiani et al., 2019 (Caligiani et al., 2019). BSF lipids (50 mg) were diluted in 0.8 mL of deuterated chloroform (CDCL3) in a 5 mm glass tube for NMR analysis. NMR spectra were registered on a Bruker Avance III 400 MHz NMR Spectrometer (Bruker BioSpin, Rheinstetten, Karlsruhe, Germany) operating at a magnetic field-strength of 9.4 T. Spectra were acquired at 298 K, with 32 K complex points, using a 90° pulse length and 3 s of relaxation delay (d1). 128 scans were acquired with a spectral width of 9595.8 Hz and an acquisition time of 1.707 s. The relaxation delay and acquisition time allow the complete relaxation of the protons, allowing their integrals for quantitative purposes. The whole zone ranging from 0.87 to 2.90 ppm plus signal centred at 5.35 ppm were used as determinant indicative of total fatty acid moles, both free and bound. For the glycerol esters, the specific signals reported in Caligiani et al., 2019

were integrated and integrals normalized for the number of hydrogen contributing to the specific signal. In the case of fatty acid integral, the mean number of hydrogen in fatty acids was inferred from the mean fatty acid composition obtained by gas chromatographic analysis, and it was found as 27.7. The normalized areas obtained were converted as relative molar percentages. To determine the molar percentage of free fatty acids, the total fatty acid molar percentages was subtracted of the contribution of fatty acids bound to TG, DG and MG**.**

4.2.11 Determination of monoglycerides of lauric acid by GC-MS

The evaluation of the level of integrity of the lipid fraction of the insect samples, mainly focalized on the Crude fat was extracted with the method of (FOLCH et al., 1957), using dichloromethane instead of chloroform, as suggested by (Cequier-Sánchez et al., 2008), with some modifications. Briefly, 5gr of each grinded BSF sample were at first mixed with 15 mL of methanol for 10 minutes. Subsequently, 30 mL of dichloromethane were added, and the mixture were blended for 40 minutes more. The samples were transferred by filtration into 50 mL test tube and an aqueous solution of KCl (0.88%, w/v) was added in an amount equal to ¼ of the total filtrate volume. The mixtures were vigorously shaken and vortexed for few seconds. The agitation was followed by centrifugation at 3900 rpm at 4 °C for 30 min. After centrifugation the inferior organic phase containing lipids was saved. To be sure to remove all the water-soluble compounds present in the sample and recover all the fats, the organic phase was again mixed with an amount equal at ¼ of the total organic phase obtained of a solution of methanol/aqueous saline solution (KCL 0.88%, w/v) 1:1 and centrifuged at 4°C for 30 min at 3900 rpm. The small upper aqueous phase was removed and the organic phase on the bottom containing the lipid fraction was filtered and evaporated by using rotavapor. BSF oil samples were kept at −20 °C and in the dark until analysis. Before the instrumental analysis, 30 mg of each extract were mixed with 0,5 mL of DMF, 0,5 mL of hexamethyldisilazane and 0,3 mL of chlorotrimethylsilane. The mixture was heated for few minutes. 4 mL of hexane were added to each sample to match the linearity range of the GC-MS instrument. The solutions were split injected (1µL) on an Agilent 7820A gas-chromatograph (Agilent Technologies, Inc., Shanghai, China) carrying a

Supelco SLB-5 capillary column (30m, i.d 25 mm, Supelco, Bellafonte, USA) coupled to an Agilent 5977B single quadrupole mass spectrometer (Agilent Technologies, Inc., Shanghai, China). The carrier gas was helium (1 mL/min), injector and detector temperatures were kept at 280 °C, while oven temperature was programmed from 60 to 280 °C at 20 °C/min.

4.2.12 Determination of integrity of the protein fraction after fermentation by UPLC-ESI/MS

To evaluate the integrity of the proteins and to identify the peptides potentially generated after fermentation, an extraction of the water-soluble compounds was performed following the method described by (Bottari et al., 2017), with some modifications. 4,5 mL of HCl 0.1 M were added to one gram of grinded samples of BSF prepupa, puparia and adults. Samples were homogenized for 1 min using an UltraTurrax (IKA T50 basic, Staufen, Germany). 0.25 mL of phenylalanyl-phenylalanine (Phe-Phe) 1 mM in water was added to each sample and used as internal standard. Acid conditions generated a precipitation of the insoluble proteins, removed by centrifugation (30 min at 4 °C at 3900 rpm). The supernatant containing the water-soluble compounds was filtered through paper filter and extracted three times with diethyl ether to remove the fat component. Ether residues were removed with a rotavapor, and the residual solutions were centrifuged again for 15 min at 4°C at 3900 rpm. Subsequently 1,5 mL of supernatant was collected and stored at -20°C until use. Samples were separated by a reverse phase column (Acquity UPLC BEH 300 C18, 1.7 µm, 2.1 × 150 mm equipped with a Acquity UPLC BEH C18 VanGuard Pre-column, 300 Å, 1.7 µm, 2.1 × 5 mm, Waters) in an UPLC system coupled with ESI- MS (UPLC Acquity with a single quadrupole detector SQD, Waters). Elution was performed with the following gradient of eluent A (water with 0.1% formic acid and 0.2% acetonitrile) and eluent B (acetonitrile with 0.1% formic acid): 0–7 min, 100% A; 7–50 min, from 100% A to 50% A; 50–52.6 min, 50% A; 52.6–53 min, from 50% A to 0% A; 53–58.2 min, 0% A; 58.2–59 min, from 0% A to 100% A; 59–72 min, 100% A. The UPLC-ESI/MS parameters were: flow 0.2 mL/min; analysis time 72 min; column temperature 35 °C; sample temperature 18 °C; injection volume 10 µL for water soluble extracts; acquisition time 0–58.2 min; ionization type: positive ion mode; capillary voltage 3.2 kV; cone voltage 30 V; source temperature

150 °C; desolvation temperature 350 °C; cone gas flow 100 L/h; desolvation gas flow 650 L/h. Samples were analyzed in the Full Scan mode, with a scan range of 100–2000 m/z. The ions of interest, corresponding to oligopeptides were integrated using MassLynx software (4.0) and semi-quantified using Phe-Phe area. The ratio between the chromatographic peak of the peptide and that of Phe-Phe did not yield absolute peptide concentration but allowed the comparison of the same peptide in different samples.

4.2.13 Peptides identification by High Resolution Mass Spectrometry on LTQ-Orbitrap Instrument

The same liquid protein extracts used for the determination of integrity of the protein fraction after fermentation were dried and reconstituted with 50 µL of 0.2% formic acid solution for mass spectrometric analysis. High resolution mass spectrometry was performed on the samples for peptide identification using a µHPLC DIONEX Ultimate3000 interfaced with an LTQ-Orbitrap XL Thermo Fisher Scientific. Column: Jupiter C18 4 µ, Proteo 90 Å 150 × 0.30 mm, Phenomenex; eluent A: water +0.1% formic acid; eluent B: acetonitrile +0.1% formic acid; flow: 5 µL/min, gradient: 0–4 min from 100% A to 95% A, 4–60 min from 95% A to 50% A, 60–62 min from 50% A to 10% A, 62–72 min 10% A, 72–74 min from 10% A to 95% A, 74–90 min 95% A; analysis time (min): 90; column temperature (°C): 30; injection volume (µL): 5; acquisition time (min): 0–75; ionization mode: ESI+; scan range (m/z): 200–1800; source voltage (kV): 3.5; capillary voltage (kV): 35; source temperature (°C): 275. Scan event details: (Fourier transform) FTMS + p res = 30,000 or (250.0–2000.0); (ion trap) ITMS + c Dep MS/MS Most intense ion form; activation type: CID; isolation width: 2.00; normalized coll. energy: 35.0; default charge state: 2; activation Q: 0.250; activation time: 30.000; dynamic exclusion enabled; repeat count: 2; repeat duration (s): 10.00; exclusion duration (s):30.00. Charge state rejection: enabled; unassigned charge states: rejected; charge state 1: rejected; charge state 2: not rejected; charge state 3: not rejected; charge states 4+: not rejected; ion signal threshold: 10,000. Protein identification was performed by using PEAKS software (Bioinformatics Solutions Inc) and INSECTA (UniProt) database. Positive hits for protein identification were arbitrarily set for all those proteins identified by the program with a score (expressed as -10lgP) >50, all those peptides with a

score (-10lgP) >20 and ppm in the range ± 6, since such value should reduce the risk of false positives to zero.

4.3. Results and discussion

4.3.1 Preliminary data on fermentation and antimicrobial activity

In the preliminary work of Hadj Saadoun et al. (2020), fermentation of insect waste (puparia and adult insects) and prepupae was carried out by inoculating two different LAB strains, L. plantarum (285) and L. rhamnosus (1473), at the concentration of 7 Log CFU/g. After 72 h of incubation at the optimal temperature for each species (30 °C for L. plantarum and 37 °C L. rhamnosus), the strains showed different growth ability. L. plantarum was able to grow in puparia and adults with an average of 2 Log CFU/g while in prepupae there was an initial decrease from the original inoculum of about 2 Log CFU/g (T0). Conversely, L. rhamnosus were able to grow only in puparia, with an increase of ca. 2 Log CFU/g, while in prepupae a decrease of about 5 Log CFU/g from initial inoculum was recorded. In adults, although a reduction occurred after inoculum, the growth of L. rhamnosus was restored up to 3 Log CFU/g at 72 h.

Table 4.1 Bacterial counts of two different lactic acid bacteria (LAB) strains in prepupae, puparia, and adults of BSF after initial inoculum and after 72 h of fermentation. Data are reported as Log CFU/g (average values ± standard deviation).

	L. plantarum 285			*L. rhamnosus* 1473		
	T_0	T_{72}	$\Delta (T_{72} – T_0)$	T_0	T_{72}	$\Delta (T_{72} - T_0)$
Prepupae	5.61±0.68	7.80±0.57	2.19	4.81±1.30	2.64±0.36	-2.17
Puparia	7.19±0.28	9.36±0.10	2.17	7.15±0.64	9.11±0.29	1.96
Dead Adults	6.65±0.43	8.84±0.48	2.19	4.29±0.81	7.33±0.49	3.04

Our data demonstrated two main trends: i) a bacterial growth was observed after LAB inoculum in puparia ii) a decrease of bacterial count after inoculum was detected and, after 72 hours of incubation, cell growth was restored or a further cell decrease was reached. Thus, interestingly we observed an intrinsic antimicrobial activity of substrates prepupae and dead adults against LAB.

The *in vitro* antimicrobial activity was carried out by microbial challenge tests, using three pathogenic strains belonging to L. monocytogenes, Salmonella spp., E. coli. The antimicrobial activities of the insect waste (puparia and bodies of dead adult insects) and prepupae were determined by evaluating the growth of different pathogens in fermented and unfermented samples on a selective medium for each pathogen.

Unfermented samples inoculated with Listeria monocytogenes LMG 21264 (Figure 4.1) highlight a significant microbial count reduction of about 3 Log CFU/g ($p<0.05$) after the original inocula (ca. 6 Log CFU/g) in prepupae and adults. This trend is not maintained during incubation because in prepupae there was an increase in concentration after 24h and 48h, while in adult samples, after an initial decrease there was an increase of 4 Log CFU/g at 48h. In puparia growth of Listeria monocytogenes is recorded after inoculum and after 24h, while there is a slight decrease after 48h (ca. 0.90 Log CFU/g) probably due to loss of viability. Moving to all fermented samples, a rapid reduction in total viable cells under the detection limit (1 Log CFU/g) was reached after inoculum (T0) and maintained for 48 h of incubation. Interestingly, no correlation was detected between the ability of the strains to grow in insect waste during fermentation and the antimicrobial activity.

Regarding microbial challenge test with Salmonella Rissen (figure 4.2) a different trend was observed. In this case, unfermented samples didn't show a decrease in pathogen load. The concentration after inoculum is nearly about the original inocula, ca. 7 Log CFU/g, and an increase of about 1 Log CFU/g was recorded after 24 h.

Differently from the microbial challenge test with Listeria monocytogenes, only three of the fermented samples show an antimicrobial activity: puparia fermented with both LAB strains and adults fermented with L. plantarum. In particular, a significant reduction ($p<0.05$) of the concentration of pathogens is recorded after inoculum, but a further drop

was shown after 24 h until 48 h. On the other hand, fermented prepupae and adults fermented with L. rhamnosus revealed similar behaviour to unfermented insects. For these samples a correlation ($r = 0.69$; $p<0.05$) between microbial counts and antimicrobial activities was detected.

Finally, considering Escherichia coli K88, a decrease of its microbial count was observed at T0 in all unfermented substrates, but after 24 h and 48 h, the growth was restored (figure 4.3). Overall, in fermented prepupae and adults fermented with L. rhamnosus it was not observed a relevant antimicrobial activity while for the other fermented samples (Puparia 1473, Puparia 285, Dead Adults 285) a trend similar to Salmonella was showed.

Interestingly, after lactic acid fermentation, the antimicrobial activity significantly increased. To note, a strong correlation between the ability to grow and the reduction of pathogens was recorded. Indeed, puparia fermented with L. plantarum and L. rhamnosus and adults fermented with L. plantarum showed the highest LAB growth during fermentation and the highest antimicrobial activity against Salmonella Rissen and Escherichia coli k88. On the other hand, this trend is not recorded in microbial challenge test with Listeria monocytogenes where all samples showed antimicrobial activity.

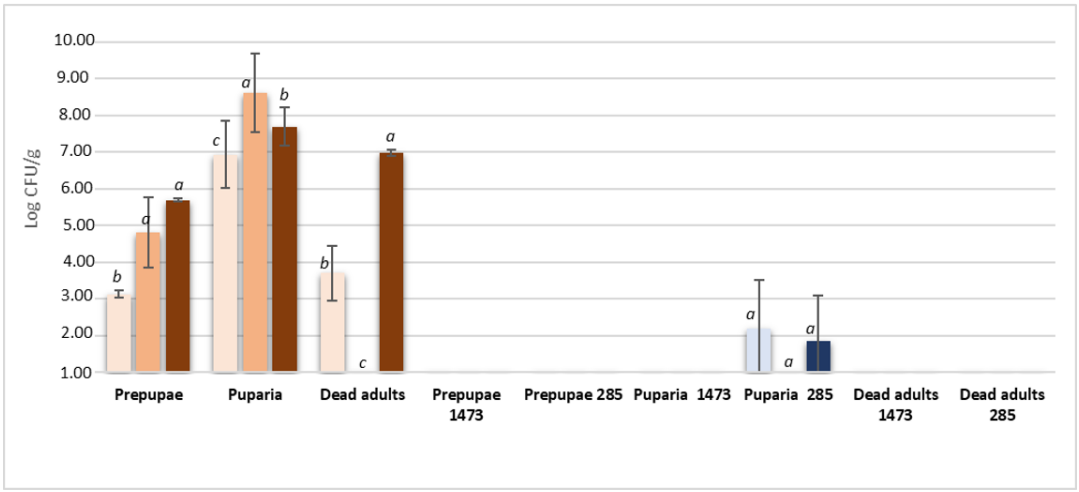

Figure 4.1. Growth of Listeria monocytogenes LMG 21264 on fermented/unfermented insect waste after inoculum (first line/light colour), 24h (second line/medium colour), and 48h (third line/dark colour). Starting inoculum 6 Log CFU/g. Letters a-c mark significant ($p<0.05$) differences among the samples. 1473: fermented with L. rhamnosus; 285: fermented with L. plantarum

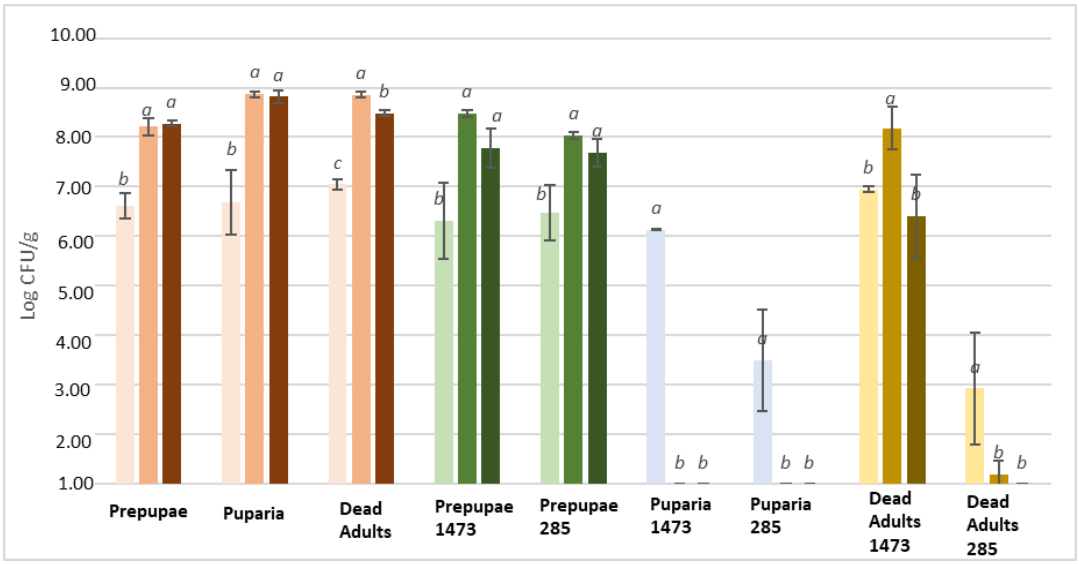

Figure 4.2 Growth of Salmonella Rissen on fermented/unfermented insect waste after inoculum (first line/light colour), 24 h (second line/medium colour), and 48 h (third line/dark colour). Starting inoculum 7 Log CFU/g. Letters a-c mark significant ($p<0.05$) differences among the samples. 1473: fermented with L. rhamnosus; 285: fermented with L. plantarum

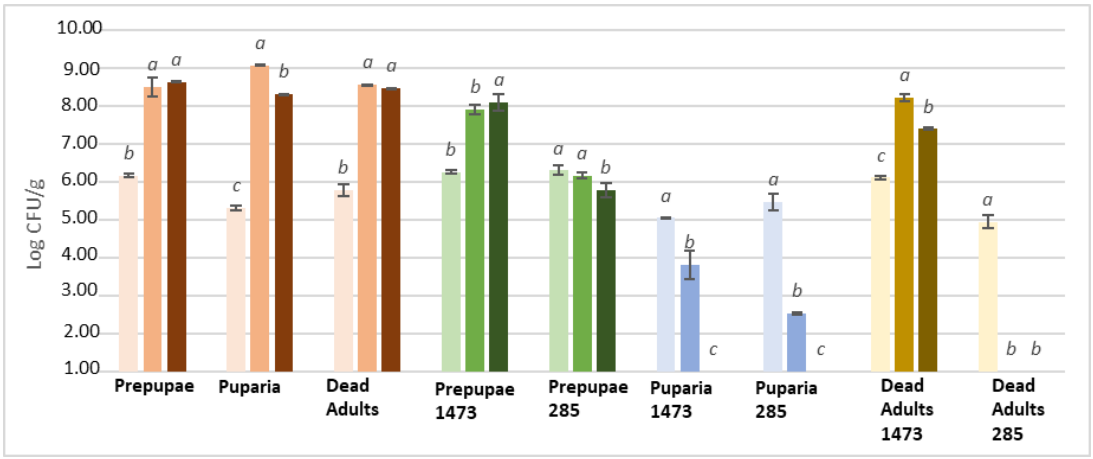

Figure 4.3. Growth of Escherichia coli k88 on fermented/unfermented insect waste after inoculum (first line/light colour), 24 h (second line/medium colour), and 48 h (third line/dark colour). Starting inoculum 7 Log CFU/g. Letters a-c mark significant ($p<0.05$) differences among the samples. 1473: fermented with L. rhamnosus; 285: fermented with L. plantarum

4.3.2 Fermentation and proximate composition

The preliminary analysis on LAB fermented insect biomasses showed that puparia and adults are fermentable and after fermentation, they show antimicrobial activity. In addition, on a molecular level, we observed a shift in lipid and protein composition induced by LAB, suggesting that the fermentation caused important changes in the molecular composition of the biomass analysed. Following the encouraging results of this work, to deepen the knowledge about the insect materials molecular composition and the insect components responsible for the antimicrobial activity, a new fermentation of the BSF prepupae and derived biomasses was carried out maintaining the same conditions used by Hadj Saadoun et al. (2020). In this work, LAB strains confirmed the growth abilities on the different substrates previously observed. *L. rhamnosus* (1473) and *L. plantarum* (285) showed the best growth capacity on puparia and on adult sample. In both cases, after 72 h of incubation, an average increase of 2.5 Log CFU/g was achieved. On the contrary, BSF prepupa as fermentation substrate induced a reduction of average 3 Log CFU/g for *L. rhamnosus*. To better understand these results, a detailed investigation on molecular changes induced by the fermentation was carried out. As a first approach, the proximate composition in terms of moisture, protein, crude fat, chitin, and ash, was performed and reported in Table 4.2.

Table 4.2 Proximate composition of unfermented and fermented BSF adults, puparia, and prepupae with two different LAB strains *Significance (<0,05) of each fermented sample respect its correspondent unfermented material (t-test comparation). **Values are expressed on dry matter basis (sugar free) and are the result of four replicate analysis.

Composition (%)	***AD_Unf***	***PUP_Unf***	***PRE_Unf***	***AD_1473***	***PUP_1473***	***PRE_1473***	***AD_285***	***PUP_285***	***PRE_285***
Lipid (Soxhlet)*	25,8±0,5	12,5±1,8	23,6±0,4	26,2±0,4	12,9±0,2	17,9 ±0,3	24,2±0,6 *	8,8±0,7	25,6±0,9
Proteins, from total AA(UPLC/ESI-MS)*	59,6±1,8	39,5±2,1	59,6±1,0	59,9±0,5	38,5±0,1	61,5±0,3	61,0±0,2	36,4±0,5	55,4±0,6
Chitin (UPLC/ESI-MS Determination of Glucosamine)*	8,3±0,2	23,8±0,3	10,0±0,4	8,7±0,1	22,0±0,3*	9,3±0,3	8,17±0,01	26,0±0,5	8,9±0,6
Ash (Oven 550 °C)*	6,1±0,1	23,9±0,1	6,6±0,0	4,9±0,1 *	26,4±0,3	11,1±0,1 *	6,5±0,1	28,5±0,1	9,8±0,1 *

Regarding the starting materials (unfermented samples), BSF adults and prepupae resulted to be richer in lipids and protein as compared to puparia. On the other hand, as expected, chitin and ashes were higher in puparia, the chitinous shell of insects. Being the insect exuvia, in fact, it is supposed to be composed mainly by chitin and minerals while, as known, the fat and protein accumulation takes place largely inside the insect's body (Liu et al., 2017; D. G.A.B. Oonincx et al., 2015). Focusing on the differences before and after fermentation, as a whole in all the samples no particular modification in the proximate chemical composition occurred. Regarding lipid fraction the only significative difference is related to the adult sample fermented with *L. plantarum,* where the lipid content resulted to be significatively slightly lower respect to the unfermented sample. For the protein fraction no significant differences were found in the protein content in all the samples before and after fermentation, independently by the LAB strain utilized.

Chitin content, reaching the highest levels in puparia, remains generally constant after the fermentation, with the only significant difference in the chitin value found in puparia fermented by *L. rhamnosus (*22.0 ± 0.3%) respect that one in the unfermented puparia (23.8 ± 0.3 %). Regarding ashes, BSF prepupa and adult show a significant difference with respect to the starting material when fermented respectively with both strains and just with *L. plantarum*. The results obtained are completely in line with those ones reached for the same fermentation biomasses in previous investigation (Hadj Saadoun et al., 2020), confirming that the main compositional differences are related to different insect material used as substrates of fermentation, while only slight effects can be attributed to the fermentation and to the different LAB strains used. Despite this, it is generally known that solid state fermentation is a biotechnological process able to change the nutritional properties of food beyond the proximate composition (Giacometti & Buretić-Tomljanović, 2017). Thus, the fermented lipid, protein and chitin fractions were further analysed to explore the differences in respect to the insect starting materials to a deeper molecular level, also using -*omics* approaches

4.3.3 BSF lipid fraction

4.3.3.1 Variations of Fatty acid profile after fermentation

GC-MS analysis (paragraph 4.2.9) on the fat extracted by Soxhlet was performed in order to compare the fatty acid profiles of the unfermented and fermented BSF samples. The detailed fatty acid composition of BSF prepupae and derived biomasses before and after LAB incubations had already been reported, indicating a clear shift, after fermentation, from a typical lipid composition of insects (rich in lauric acid) to a mixed composition of both insects and lactic acid bacteria (characterized by long chain fatty acids). (Hadj Saadoun et al., 2020).

Data of fatty acid redistribution were re-elaborated for evaluating the lipid fraction of fermented insect biomasses from a nutritional point of view. The differences in the fatty acid composition in terms of nutritional value are reported in Table 4.3. Here were reported the classic indices generally used to assess the nutritional value of food lipid fraction, such as the sum of saturated fatty acids (SFA), of monounsaturated fatty acid (MUFA), of polyunsaturated fatty acid (PUFA), the PUFA/SFA ratio, and the oleic and the linoleic acid amount, in addition with the most common fatty acids in BSF matrix (i.e. Lauric acid) (Chen & Liu, 2020).

Table 4.3. Major fatty acid composition, fatty acid partial sums and nutritional ratio of BSF adults, puparia and prepupae unfermented and fermented by L. rhamnosus (1473) and L. plantarum (285). Values are expressed as mean ± SD of relative percentages on total fat. SFA: Saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; LA: Linoleic acid; CLA: sum of conjugated linoleic acid isomers. *Significance (<0,05) of each fermented sample respect its correspondent unfermented material (t-test).

	AD_Unf	AD_1473	AD_285	PUP_Unf	PUP_1473	PUP_285	PRE_Unf	PRE_1473	PRE_285
Lauric acid	44.1 ± 0.4	31.6 ± 0.1*	33.7 ± 0.9*	37.2 ± 0.1	33.7 ± 1.3	30.2 ± 0.5*	47.1 ± 0.4	28.6 ± 1.2*	27.2 ± 0.6*
Palmitic acid	11.5 ± 0.3	12.5 ± 0.2	13.7 ± 0.3*	13.7 ± 0.2	17.9 ± 0.8	24.3 ± 1.3*	14.3 ± 0.2	12.9 ± 0.4	12.7 ± 0.3
SFA	73.4 ± 0.1	64.1 ± 0.1*	67.1 ± 0.2*	75.1 ± 0.1	76.5 ± 0.3	78.3 ± 0.3	80.9 ± 0.1	62.3 ± 0.3*	59.9 ± 0.1*
oleic acid	10.4 ± 0.1	8.7 ± 0.0*	6.6 ± 0.2*	11.7 ± 0.0	6.5 ± 0.3*	6.4 ± 0.1*	6.8 ± 0.0	9.1 ± 0.1*	8.6 ± 0.1*
MUFA	13.7 ± 0.0	14.5 ± 0.0*	14.4 ± 0.0	15.4 ± 0.0	10.9 ± 0.0*	10.0 ± 0.1*	9.3 ± 0.0	15.9 ± 0.1*	15.8 ± 0.0*

LA	12.1 ± 0.2	20.9 ± 0.3*	18.2 ± 0.2*	8.0 ± 0.3	11.7 ± 0.1*	11.3 ± 1.6	8.9 ± 0.3	20.1 ± 1.1*	22.6 ± 0.2*
CLA	0.0 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.0 ± 0.1	0.8 ± 0.0*	0.3 ± 0.1	0.2 ± 0.1	1.7 ± 0.2*	1.6 ± 0.0*
PUFA	12.1 ± 0.2	21.2 ± 0.2*	18.4 ± 0.2*	8.0 ± 0.2	12.5 ± 0.1*	11.6 ± 0.9	9.1 ± 0.2	21.8 ± 0.6*	24.2 ± 0.1*
PUFA/SFA	0.2	0.3*	0.3*	0.1	0.2*	0.1	0.1	0.4*	0.4*

A significant reduction in the total saturated fatty acids after fermentation with both LAB strains can be noted in BSF adult and prepupa samples. At the same time, with just a few exceptions, significantly increased levels of monounsaturated fatty acids in general were observed in BSF prepupae and derived by-products after LAB incubation with respect to the corresponding unfermented material. More specifically, among monoinsaturated fats, oleic acid (C18:1 ω9) increases significantly after fermentation only in prepupa samples, while greatly decreases in fermented BSF adults and puparia. The sum of polyunsaturated fatty acids as well grew significatively after fermentation, with the only exception of PUP_285. In particular, the percentages of linoleic acid (C18:2 ω6) is almost double in all cases after fermentation, while the conjugated linoleic acid (CLA) showed a significative increment expecially in both fermented prepupa samples and in puparia fermented with *L. rhamnosus*. Black soldier fly is known to be one of the most highly rated among insect species for animal and future human nutrition due to the high biological value of its body nutritional composition (Meneguz et al., 2018). However, it is known that BSF fat it is particulary rich in saturated fatty acids. Among these the most representative are lauric acid (C12:0) and palmitic acid (C16:0), that generally correspond respectively to the 40% and 15% the of total fatty acids (Y. B. Kim et al., 2020). Total monounsaturated fatty acids are instead present in lower concentrations, followed by the polyunsaturared. Although it has been demonstrated that BSF lipid fraction, if part of a animal diet, has a positive influence on intestinal functions, antioxidant and immunomodulatory powers (Y. B. Kim et al., 2020), it is not able to provide high concentrations of unsaturated fatty acids. It is common knowledge that monounsaturated and polyunsaturated fatty acids are highly recommended in a human diet for their numerous positive effects on health and wellness. Compared with saturated fatty acids, these have shown protective effects against cardiovascular diseases, atherosclerosis and positive modulatory properties on lipid metabolism (Lada & Rudel, 2003). Based on the results obtained in this work, fermentation leads to a redistribution of fatty acids that which results in a significantly increased PUFA/SFA ratio, enriching the nutritional profile of BSF oil of functional fatty acids.

Changes observed here in the nutritional profile of BSF fats are in accordance with many works that show how LAB fermentation could modify the positional distribution and proportions of fatty acids on different food sources, leading to enanched lipid compositions (Vieira et al., 2015). The ability of the LAB to increase the content of long chain fatty acids and to create unsaturations has also often been observed (Molina et al., 2013; Ravyts et al., 2012; Xiao et al., 2013). Vieira et al., 2015 showed that fermentation of matrices rich in lipids might lead to higher production of polyunsaturated fatty acids. This point can explain why the puparia, poor in lipids, is the sample with the lowest production of unsaturated fatty acids. Hence, the increase in oleic acid and unsaturated fatty acids followed by the decrease of saturated fatty acids observed in these samples can be considered as a natural consequence of the fermentation and the nature of the samples. As a whole, even if the BSF is already considered one of the most promising alternative to the conventional food/feed sources for its optimal nutritional composition, in this case, the fermentation seems to enrich even more the substrate with a nutritional benefits in terms of fatty acid composition.

4.3.3.2 Distribution of Lipid classes determined by ^{1}H NMR

With the aim of investigating if, beside fatty acid composition, some changes occurred after fermentation in lipid classes distribution, a 1H NMR lipidomic approach was applied on BSF prepupa and derived biomasses, before and after fermentation. Thus, 1H NMR analysis on crude fat extracted by Folch procedure (paragraph 4.2.11) was carried out and the triacylglycerols (TG), diacylglycerols, (DG), monoacylglycerols (MG) and free fatty acid (FFA) content was determined. The relative molar percentages of the different lipid classes are showed in Table 4.4. The ^{1}H NMR spectra related to the unfermented and fermented with the two LABs BSF adult are reported in figure 4.4, as example.

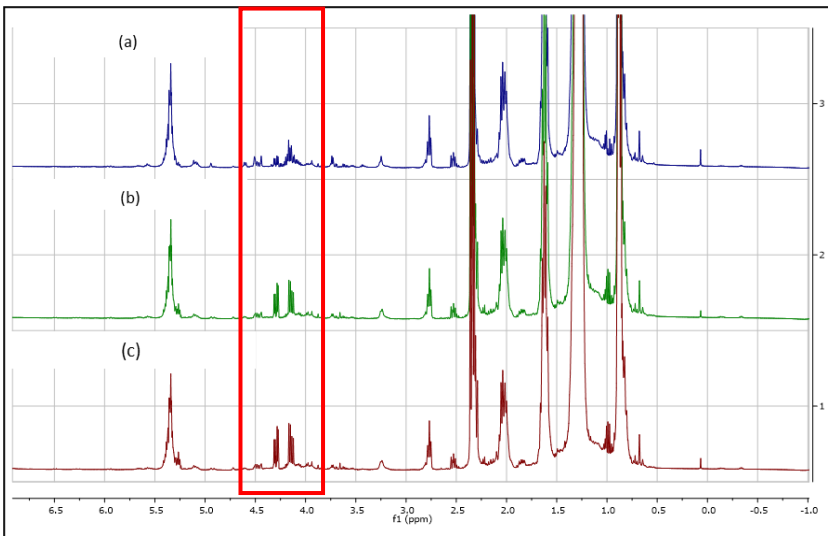

Figure 4.4: 1H NMR spectra of lipid fraction of BSF adult flies unfermented (a) and fermented by L. Rhamnosus (b) and L. Plantarum (c). The frame indicates the triglycerides' area.

Table 4.4. Relative molar percentages determined by 1H NMR of the different glycerides and free fatty acids present in BSF oil from BSF adults, puparia and prepupae, unfermented and fermented with two LAB strains. CV% < 5%. Abbreviations: TG: triglyceride; 1,2-DG: 1,2-diglyceride; 1,3-DG: 1,3-diglyceride; 2-MG: 2-monoglyceride; 1-MG: 1-monoglyceride; FFA: free fatty acid.

metabolite	AD_Unf	AD_1473	AD_285	PUP_Unf	PUP_1473	PUP_285	PRE_Unf	PRE_1473	PRE_285
1,2DG	3.32	2.2	2.11	2.17	7.56	5.41	0.26	0	0.72
2MG	0.31	0.03	0	0	0	0	0	0	0.08
TG	2	4.99	6.52	0.71	1.51	0.64	0.15	0	0.57
1,3DG	1.23	1.27	1.43	0.71	0.85	0.55	0.06	0.15	0.64
1MG	3.71	4.78	5.57	4.91	8.56	6.35	0.43	0.63	2.96
free fatty acids	89.44	86.74	84.36	91.5	81.52	87.05	99.1	99.22	95.03

Even if from the gross composition no significant variations were observed in the total lipid content of insect samples before and after fermentation, the lipidomic approach highlighted some compositional changes. The greatest effect of the fermentation was found on the content of triglycerides. Comparing fermented and unfermented spectra (figure 4.4), in fact, the main difference is in the relative abundance of the signals related to the triglycerides (red box), much higher in the fermented samples than in the unfermented ones. In this case also differences among the LAB strains used are present. For instance, the content of triglycerides in the BSF adults and puparia reaches higher values when fermented respectively with plantarum (6.52%) and rhamnosus (1.51%) respect to the corresponding unfermented samples (2% for AD_Unf and 0.71% for PUP_Unf). Also monoglyceride and diglyceride content was affected by the fermentation treatment and their content was in general higher in the fermented samples rather than in the unfermented ones. Unfermented sample resulted on the contrary richest in free fatty acids. The free fatty acids molar percentage maintained high levels (higher than 80%) also after the two different fermentation processes. The high free fatty acids amount in BSF samples found explanation in the strong lipase activity generated inside the insect biomass during insect killing by freezing, already demonstrated in other studies (Caligiani et al., 2018). The differences found after fermentation suggest that LABs are able to carry out a biosynthesis of glycerol esters using as starting material the free fatty acids naturally present in the lipid fraction of BSF.

Targeted determination of monoglyceride of lauric acid. It is known that Lactic acid bacteria fermentation can generate in raw substrates new compounds, exerting numerous bioactivities, including antimicrobial (Sadh, Kumar, et al., 2018; Septembre-Malaterre et al., 2018). Lauric acid, of which BSF is particularly rich (Caligiani et al., 2018), among all fatty acids is the strongest in the growth inactivation of Gram-positive and Gram-negative such as respectively *Staphylococcus aureus* and *E. coli* bacteria (Khoramnia et al., 2013). Many studies showed the same antimicrobial effect for monoglycerides and diglycerides of medium chain fatty acids (including C12:0) (Bergsson et al., 2001; Ruzin & Novick, 2000; Schiavone et al., 2017). Since ^{1}H NMR analysis on BSF lipid extracts showed, after

fermentation, a decrease in free fatty acids and an increase in fatty acid esters including mono and diglycerides, it was supposed a use of fatty acids by LAB metabolism directed to the synthesis of esters, such as the lauric acid monoglyceride. This is one of the most studied among all the fatty acid esters for its known bactericidal and antifungal activity (Dayrit, 2015). To exactly determine this compound, here for the first time a GC-MS analysis (paragraph 4.2.11) on the same lipid extracts used for the NMR was performed. Despite this hypothesis, no signals associated with the presence of the monoglyceride of lauric acid or other known antimicrobial esters were recognized (data not shown).

4.3.4 BSF protein fraction

4.3.4.1 Variations of total Amino Acid profile after fermentation

To strength the preliminary data on protein fraction of fermented BSF reported previously (Hadj Saadoun et al., 2020), here the complete amino acid profile of BSF samples before and after fermentation was determined by UPLC-ESI/MS analysis.

Complete data on the total Amino acid composition of insect samples are reported in Table S 4.1 of the supplementary material. Total amino acid amount has already been reported in Table 4.2. The variation in the total amino acid distribution between BSF fermented and unfermented expressed in g AA/100g AA is reported in figure 4.5, where the graph baseline represents the unfermented sample and the bars indicated how total amino acid composition changes following fermentation with the two different LAB strains.

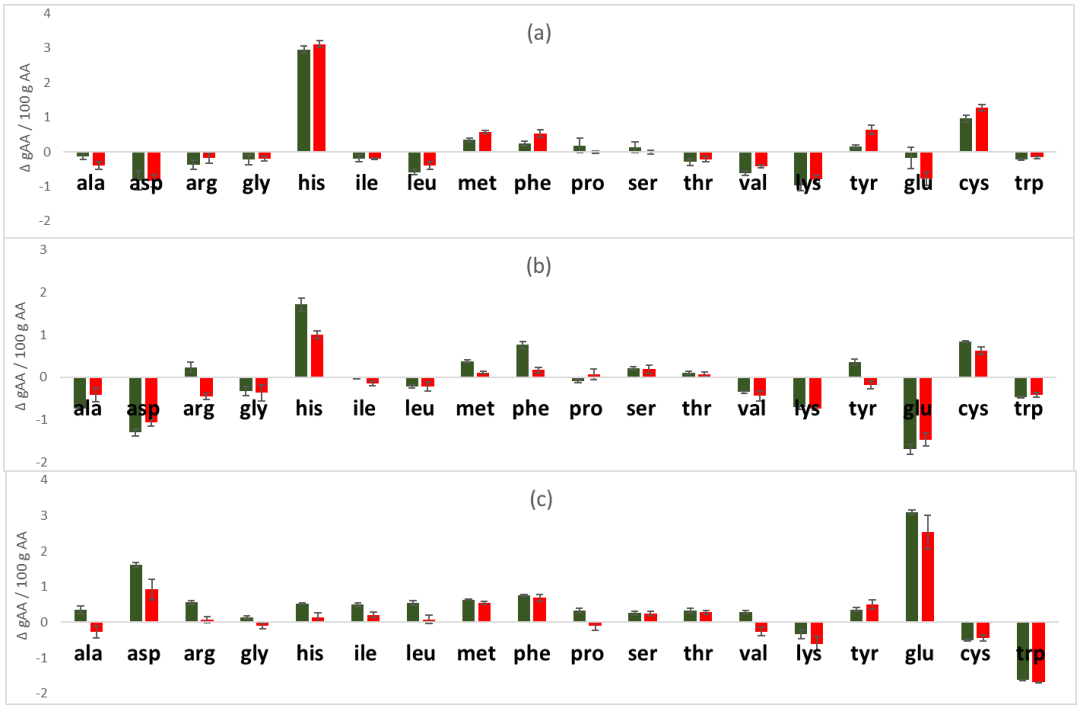

Figure 4.5 Variations of total Amino Acid distribution, expressed as relative percentage, in fermented by L. rhamnosus (first line)/ fermented by L. plantarum (second line) BSF adults (a), puparia (b), and prepupa (c) compared to corresponding unfermented sample (graph baseline).

Results are generally in line with the previous study: even if from the gross composition the total amount of proteins remained the same before and after fermentation (see above Table 4.2), looking at the amino acid composition it is clear a redistribution occurred in the protein fraction. However, compared to the work of Hadj Saadoun et al., 2020, the amino acid distribution after fermentation in the present BSF samples is slightly different. Hadj Saadoun et al., 2020 reported a variation of the amino acids redistribution also depending on the different LAB strain used for the fermentation of BSF prepupae and derived by-products. In the present study, only slight effects can be attribute to the different LAB strains. As it is possible to see from the figure 4.5, the *L. rhamnosus* and *L. plantarum*, for equal BSF substrate, exhibit the same behaviour, suggesting a similar protein metabolism. On the other hand, it is clear the fermentative effect on the amino acids' distribution depends on the insect substrate. While in the previous work of Hadj Saadoun et al., 2020, the amino acid distribution was different for each substrate, here the effect of the LAB

growth was very similar on the fermented BSF adults and puparia. Both presented respect to the corresponding unfermented biomasses, an enhanced amount of some essential amino acids such as histidine, cysteine, phenylalanine (only for AD_1473) and methionine (only for PUP_1473), and a significant reduction of other amino acids (alanine, asparagine, glycine, and glutamic acid) including the essential amino acids isoleucine, leucine, lysine, valine and tryptophan. Different changes were observed in BSF prepupa: in this case lysine, cysteine and tryptophan, and valine (only for *L.plantarum* strain), belonging to the class of essential amino acids, were the only ones in the profile which undergo a significative decrease after fermentation. All the others amino acids, with just few exceptions (as in the case of arginine), significantly increase in relation to unfermented. Except for phenylalanine and methionine, in the case of BSF prepupa the redistribution of the amino acid profile is consistent with that observed by Hadj Saadoun et al., 2020. Interestingly, the samples presenting the same variation in amino acid profile, BSF adults and puparia, were also those ones better fermented, while BSF prepupae (less fermented) have a different aminoacidic redistribution. Thus, it seems that the inability to ferment well is linked to the simultaneous inability to enrich the protein mixture in essential amino acids.

It was previously reported that the addition of fermentative microorganisms affects the amino acids composition of the organic substrate (Pozo-Bayón et al., 2005). Microorganisms can increase the concentration of amino acids because of protein synthesis by fermenting substrates or catabolize amino acids into different products, for example volatile compounds affecting food flavour (A et al., 2013; Babalola & Giwa, 2012; Manca De Nadra et al., 1997; Pozo-Bayón et al., 2005). It was therefore expected that the protein fraction of the samples would be influenced by LAB nitrogen metabolism. This therefore can explain why, where LAB grew better, similar changes in the amino acidic composition of the substrates were observed.

From a nutritional perspective, many of the essential amino acids increase their amount significatively following the bacterial growth in all substrates. This is a clear benefit of fermenting BSF prepupae and derived biomasses. Several results have been reported regarding the positive effect of fermentation on nutritional and total amino acid

composition of food (Khan et al., 2018; Nkhata et al., 2018). Based on these works, even if the total amount of protein is not changed an increase of essential amino acids it is observed. These discoveries are in line with our results, suggesting that insects as future human food could be nutritionally enriched using fermentation, even if the increase in some essential amino acids is associated with a significant decrease of others. Nonetheless this result does not compromise the nutritional value of the BSF protein fraction. Table 4.5 shows that all BSF samples, after fermentation maintained a positive nutritional profile in essential amino acids, which in any case satisfied the reference values suggested by FAO/WHO for human nutrition (Fuso et al., 2021).

Table 4.5: Values (expressed as mg/g protein) for essential amino acids of BSF adults, puparia and prepupae compared with the FAO protein reference for human adults (2011)

Essential amino acids	*AD_ Unf*	*AD_ 1473*	*AD_ 285*	*PUP _Unf*	*PUP_ 1473*	*PUP _285*	*PRE _Unf*	*PRE_ 1473*	*PRE _285*	*Reference Protein FAO 2011 (mg/g Protein)*
His	11	61	62	46	88	76	50	58	52	16
Thr	62	57	57	44	46	48	48	54	53	25
Val	79	69	71	102	91	96	72	77	67	40
Lys	50	34	36	23	4	4	51	45	41	48
Ile	59	56	55	46	45	45	45	53	48	30
Leu	95	86	87	89	82	89	78	87	79	61
Trp	16	12	13	36	23	26	44	16	15	6.6
Phe + Tyr	129	136	146	145	169	153	130	149	150	41
Cys + Met	37	59	67	30	60	52	53	56	55	23

4.3.4.2 Proteolytic effects of fermentation on BSF biomasses

To investigate the proteolytic ability of the LAB, a comparison between the peptide's profiles of the fermented substrates with the unfermented was done. The study was carried out using a UPLC/ESI-MS analysis. In the resulting chromatograms a semiquantitative evaluation of three classes of peptides, respectively corresponding at a molecular weight < 300 m/z, 300-500 m/z, and >500 m/z was made. A semiquantitative approach was chosen for every unique peptide identified (peptides have been identified based on their detected MW), allowing a direct comparison of the same chromatographic signal among the different residues. This allows having an immediate idea about the ability of the LAB to affect the protein fraction of BSF samples during fermentation. The full data are reported in supplementary table S 4.2. The results are summarised in figure 4.6.

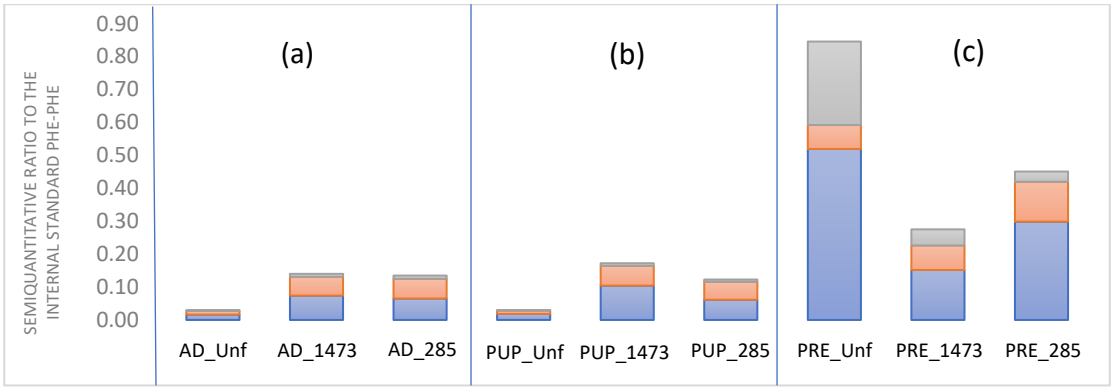

Figure 4.6 Semiquantitative percentage of < 300 m/z (blu), 300-500 m/z (orange), and >500 m/z (grey) peptides in BSF adults (a), puparia(b), and prepupae(c) unfermented and. fermented by L. rhamnosus and L. plantarum.

The unfermented prepupa is clearly the richest in peptides, which however were much reduced in fermented prepupae with both strains. It can be assumed that micro-organisms use the starting prepupa peptides as a source of nitrogen for their metabolism. No evidence of proteolytic activity was seen, and this might relate to the lower microbial growth in this substrate, as previously mentioned. On the other hand, the other insect biomasses (adults and puparia) were at the beginning very poor in peptides, while after fermentation their amount was increased, suggesting a proteolytic activity, that can be linked to the better

fermentation that occurred on insect adults and puparia with respect to prepupae. The proteolytic activity, in fact allows the microorganisms to grow successfully and rapidly. Although, as a whole, proteolytic activity found could be considered not very high. The results obtained by other proteolytic indices measured to better define the proteolytic activity of the LAB on the BSF biomasses, such as the free amino acids amount and the degree of hydrolysis (data not shown), confirmed this evidence. Analysing the evolution of free amino acids during fermentation, in fact, it turned out that the fermented BSF biomasses were characterised by a very low amount of free amino acids, ranging from 0.87 to 2.28%, and very low degree of hydrolysis (not more than 1.7%) confirming the absence of a significant proteolytic activity from LAB strains in insects. Even if Lactobacilli are documented to exert a proteolytic activity on different food sources, detailed studies have highlighted how their proteolytic system is weakest compared with that of other species, as *Lactococcus*, *Pediococcus* or *Enterococcus* genus (Aguirre et al., 2014; Daliri et al., 2018; Fadda et al., 2010; Gandhi et al., 2016; García-Cano et al., 2019; Kliche et al., 2017; Lim et al., 2019; Savijoki et al., 2006; Simitsopoulou et al., 1997). Alternatively, it might be hypothesized that some of the peptides already present in prepupae exert some antibacterial effect, effectively hampering Lactobacilli growth on these substrates.

4.3.4.3 Protein identification by high resolution mass spectrometry

In order to better understand the insect protein classes more susceptible to proteolysis by LAB, as a further step, a high-resolution mass spectrometry characterization of peptides present in fermented biomasses was carried out. In this way is possible to understand which proteins are the preferential substrate of LABs during their growth. Figure 4.7 reports the identified peptide distribution, based on their origin in the BSF adults, puparia and prepupae fermented with the two different strains. It was found that the peptides identified in the fermented BSF adults were all of muscular origin. In particular, they were myosin and actin derived peptides. On the other hand, most of the peptides present in the fermented prepupa and puparia were respectively from cuticular (such as pupal/larval cuticle proteins) and metabolic proteins. From these results, it seems that the peptide origin reflects the most abundant classes of proteins in BSF biomasses. It is known in fact

that puparium, the cuticle which cover the larval and prepupa stage before the fly metamorphosis, is particularly rich of chitin closely linked to cuticular proteins, both derived from the epidermis layer just below (Tajiri et al., 2017). Although no specific studies were found in the literature that investigates the most abundant protein classes present in the BSF adults, it is also clear that removing the exoskeleton during the transition to the adult stage of BSF prepupae will result in the richest quantity of muscle proteins compared to the prepupa body. From the results obtained by the most abundant peptides after fermentation it is evident that LABs consume the most abundant proteins present in the matrix they are fermenting, suggesting good flexibility to grow on different kinds of nitrogen sources.

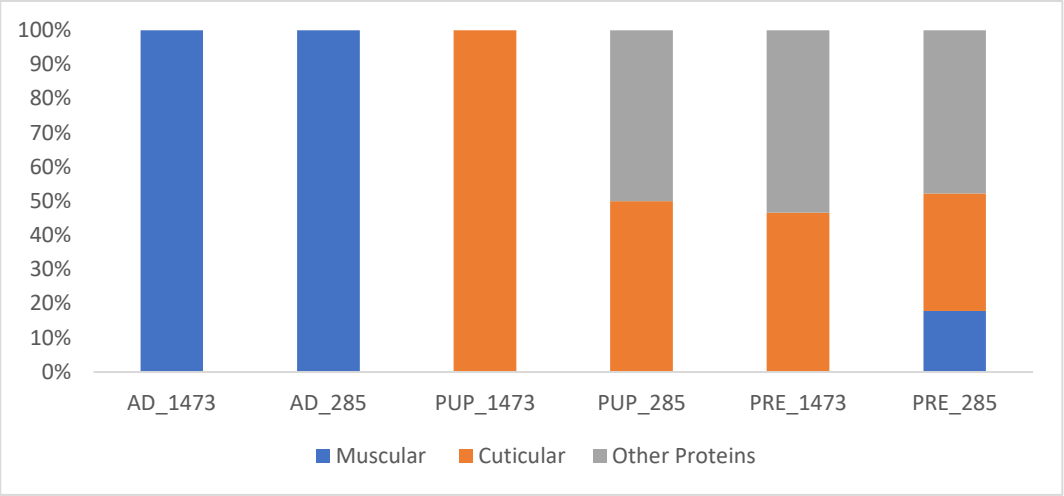

Figure 4.7 Distribution of peptides identified by High Resolution Mass Spectrometry on LTQ-Orbitrap instrument in BSF adults, puparia and prepupae fermented with L. rhamnosus and L. plantarum extracts according to their origin: muscular, cuticular or other.

4.3.5 Effect of fermentation on chitin's oligosaccharides profile

To learn more about the hydrolytic behaviour of the LAB, the potential hydrolytic activity on the insect chitin with consequent formation of Chito-oligosaccharides has been evaluated by using a UPLC-ESI-MS analysis. The chromatograms obtained from the analysis did not show the presence of chitooligomers for any insect samples. This can suggest or the absence in the LAB metabolism of chitinase or a low hydrolytic activity on chitin fraction, due to its insoluble crystalline structure. In a previous work on chitinase activity in *Lactobacillus* strains, no chitinolytic activity was observed, although the presence of chitinase coding gene in the microorganisms was confirmed (Horwh-Szanics et al., 2020). It is therefore possible even if the LAB selected for the experiment showed in their genomes the presence of different chitinases (data not shown), they were not able to express them. Still few works in literature on the *Lactobacillus* strains chitinase are present to know the reason for this lack of activity despite the presence of the LAB chitinase gene and further studies to refer to are needed.

4.4. Conclusions

Lactic acid bacteria fermentation was chosen as a means of management of insect biomasses. In this work, molecular variations following fermentation by inoculation of *L. rhamnosus* and *L. plantarum* strains in insect wastes composition were studied and compared with the starting residual material, in order to explore the LAB's ability to modify the molecular composition of the substrate. From the current investigation it was found that the fermentation actually induced a modified molecular composition of the biomasses, even if this modification was barely visible in the bulk composition. The fermented samples presented a fatty acid profile enhanced in monounsaturated and polyunsaturated fatty acids and poorer of saturated medium chain fatty acids and a modified aminoacidic profile richest of essential amino acids (at least the samples which fermented better). As a whole, no significant differences in the behaviour of the different LAB strains selected were observed. A proteolytic activity, although not pronounced, has been found in both LAB strains, but only apparent in the better fermented substrates. From this point of view, fermentation can be the way to obtain from these by-products insect-based ingredients with unique properties for feed and industry. The envisaged beneficial properties that these enhanced residues can acquire include the antioxidant, health-promoting, antimicrobial. From previous studies antimicrobial activity was found in fermented BSF insect material, especially in puparia and adults (Hadj Saadoun et al., 2020). As a conclusion, this previous investigation suggested deepening the knowledge at the molecular level of the BSF prepupa and derived biomasses, in order to elucidate the origin of antimicrobial activity. In this perspective, our investigation constitutes an excellent starting point giving us a precise overall view of the molecular changes that the substrate undergoes after fermentation. A further step will be to identify the molecules exerting the antimicrobial activity and understand their origin.

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CHAPTER 5

Inhibition of DPP-IV activity and stimulation of GLP-1 release by gastrointestinally digested Black soldier fly prepupae

Abstract

The beneficial effects on human health of an insect-based diet and in particular the regulatory ability of digested insects' proteins on the glycaemic response in humans are topics that need to be deeper investigated. In this work, we performed an in vitro study on the modulatory activity of gastrointestinal digested black soldier fly (BSF) prepupae on the enterohormone GLP-1 and on its natural inhibitor, DPP-IV and we verified whether actions intended to valorise the starting insect biomass i.e., insect optimized growth substrates and prior fermentation, can have a positive impact on human health. Our results highlight that the digested BSF proteins from all the different prepupae samples had a high stimulatory and inhibitory ability respectively on the GLP-1 secretion and the DPP-IV enzyme in the human GLUTag cell line. Gastrointestinal digestion significatively improved DPP-IV inhibitory capacity of the insect whole protein. Moreover, it was seen that optimized diets or fermentation processes preceding the digestion, in any case, did not positively affect the efficacy of the answer. BSF was already considered one of the edible insects more suitable for human consumption for its optimal nutritional profile. The biological potential and the positive effects on in vitro human glycaemic answer here found makes this species even more promising. To the best of our knowledge, this is the first time that the BSF species is studied for its effect on human glycemic regulation. Deeper investigations are suggested to confirm the promising results obtained in this study.

5.1 Introduction

Until quite recently, in most western countries, insects were just considered as pollinators, waste enrichers, natural bio controllers for harmful pest species in agriculture, at most as providers of a variety of products (i.e., honey) etc, but practically never were they taken into consideration as part of a western diet. This view was in stark contrast with the dietary cultures of other countries worldwide, where instead more than 1900 species of insects (including for example Coleoptera, Lepidoptera, Hymenoptera etc) were and are commonly consumed (Nowak et al., 2016; van Huis et al., 2013). The problem of the relevant and constant growth world population, predicted to reach 9.7 billion people during the next 30 years, raised the issue also in western societies of using insects as a food source (ONU, 2019). The principal problem of overpopulation is in fact related to the need to find an alternative source of high biological value nutrients, especially proteins, for human and animal requirements when other sources will be no longer sufficient (van Huis, 2015). In this context, insects are considered a strategic solution in terms of positive effects on both, environment, and human health. An insect large-scale rearing is far more sustainable than livestock farming, for their highest feed conversion efficiency, limited water, and land requirement, and low gas emissions. Moreover, insects are an excellent source of important fatty acids (such as linoleic, omega-6 and α-linolenic acids, omega-3), chitin, vitamins, and minerals, with the advantage of being rich in proteins having a high biological value. The protein content in the insect body can reach 70% on dry basis, higher than most plants and other commercial animal protein sources such as meat, eggs etc (Bessa et al., 2020; Jonas-Levi & Martinez, 2017; Xiaoming, C., Ying, F., Hong, Z. and Zhiyong, 2010). Among the order of diphthera (Stratiomyidae), *Hermetia illucens*, also called Black soldier fly, is one of the most promising choices for massive breeding to be used as the basis of food and feed ingredients. Numerous studies demonstrated how black soldier fly satisfy widely all specific dietary requirements for humans and farmed animals, thanks to their optimal nutritional composition (Bosch et al., 2014; Cummins et al., 2017; Spranghers et al., 2017; Virginia et al., 2016). Black soldier fly prepupa, the insect life stage most studied from a nutritional point of view, has been reported to contain (expressed on

dry mass) 32% of proteins, 37% of lipids, 19% of minerals and 9% of chitin (Caligiani et al., 2018). Since eating insects is thought to be one of the potential answers to the growing protein world demand, the interest in understanding the effects of this alternative food source on health is increasing. From the literature, it turns out that most of the studies only examine the beneficial effects of an insect-based diet on livestock health status (Borrelli et al., 2017; Marono et al., 2017). For example, numerous are the studies that showed the good support that the integration with *H*. *illucens* prepupae in a diet can have on chickens and other animals' growth and on gut health (Borrelli et al., 2017; Hale, 1973; Newton et al., 1977; Pimentel et al., 2004; Sheppard et al., 2007; St-Hilaire et al., 2007). On the contrary, there are not many studies aimed to measure health outcomes on human subjects from insect consumption (Stull, 2021). Few studies showed that their consumption can in general promote both, human and animal health, preventing or controlling health risks such as hypertension or diabetes, and reinforcing the immune system (Oonincx et al., 2019; Paul et al., 2017; Payne et al., 2016; Wathne et al., 2018; Womeni et al., 2009). As recent researches stated, some of these beneficial effects are possible because from the insect protein fraction bio-active peptides can be generated (Nongonierma & FitzGerald, 2015; Wu et al., 2015; Zielińska, Baraniak, et al., 2017; Zielińska, Karaś, et al., 2017). However, the interaction of these molecules with the gastrointestinal tract and systemic health benefits associated are topics that have not yet been adequately explored. One of the most important findings of the last years is that the enzymatic hydrolysis can generate from insect proteins, proactive peptide fragments that positively affect the postprandial glycemic response (Filippatos et al., 2014; Jao et al., 2015; Lacroix & Li-Chan, 2016; Rivero-Pino et al., 2021). From *in vitro* and *in vivo* experiments, it was observed that the hydrolysates of these novel foods can stimulate, more than other protein sources, the gut secretion of a class of hormones called incretins, including the GLP-1, that promote the insulin release and the intestinal absorption of glucose, blocking the glucagon release (Miguéns-Gómez et al., 2020). Moreover, they are found to be natural inhibitors of the dipeptidyl-peptidase IV (DPP-IV), a potent antagonist of GLP-1 enterohormone (Lacroix et al., 2019; Nongonierma et al., 2018; Rivero-Pino et al., 2021). Thus, it may be inferred that

in general edible insects, if introduced in a human diet and subjected to a complete digestion process, could be an optimal source of bioactive peptides that, thanks to their mechanism of action, could represent an alternative to the use of pharmaceutical substances in the management of metabolic diseases such as diabetes. However, no specific investigations have yet been made concerning the black soldier fly. More controlled studies are fundamental to confront and confirm these insect-derived ingredients health benefits and better assess activity of bioactive peptides potentially generated from the digestion. Thus, in order to evaluate more in-depth the beneficial effects of being waged by an insect-based diet on human health and in face of the lack of information on the effects of a specific interaction between the black soldier fly species and a human intestinal model, the aim of this work was double: at first explore the modulatory activity of the digested original insect on the enterohormone GLP-1 and on its natural inhibitor, DPP-IV. Then, to check if this effect can be affected by the insect diet or by a previous fermentation process applied to the insect biomass.

5.2 Materials and methods

5.2.1 Insect samples and proximate composition

The Black soldier flies (*Hermetia Illucens,* HI) prepupae samples, from larvae purchased from CIMI srl (Cuneo, Italy), derived from a larval rearing experiment carried out by the Applied Entomology laboratory of the University of Modena and Reggio Emilia (Italy) and from a fermentation experiment carried out by the microbiologist laboratory of university of Parma (Italy). More specifically, for the current investigation were used: a) Black soldier fly prepupae reared on a control diet, composed by 50% wheat bran, 30% alfalfa meal and 20% corn meal (CP); b) black soldier fly prepupae reared on an optimal agri-food by-products mixture in terms of breeding performances (PAgri) c) black soldier fly prepupae from control diet, incubated with two different *Lactobacillus* (LAB) strains, LAB rhamnosus (PFR) and LAB plantarum (PFP). All the insects were killed by freezing at -20°C and finely grinded for 2 minutes with IKA A10 laboratory grinder (IKA Werke GmbH & Co. KG, Staufen, Germany). The samples undergoing fermentation were prepared as described by Hadj

Saadoun et al. (2020). Briefly, prepupae, once grinded, were blended with sugar (8.5% w/w). the mix obtained was sterilized in autoclave at 121° for 20 minutes in a glass jar. 30g of BSF prepupae/sugar substrate were then collected and incubated with a final concentration of 7 Log CFU/g of each bacterial suspension for 72 h at 30 °C for L. plantarum 285 and at 37 °C for L. rhamnosus 1473. All the insect samples were lyophilized and stored at -20°C until the analysis (Barbi et al., 2020; Fuso et al., 2021; Hadj Saadoun et al., 2020). Standard procedures (AOAC, 2002) were used to evaluate the composition in moisture, lipid and ash of BSF prepupae samples. Moisture was determined in the oven at 105 °C for 24 h. To determine crude fat, an automatized Soxhlet extractor (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy) was used, using diethyl ether as extraction solvent. Ashes were determined after mineralization at 550 °C for a total time of 10 h (5 h + 5 h). From the sum of total amino acids released after acid hydrolysis, prepupae total protein content was calculated, following the method described by Caligiani et al. (2018). Chitin and other compounds' content were calculated by difference to the quantity of proteins, ashes, and lipids.

The digested Buffalo worms (*Alphitobius Diaperinus*) sample tested simultaneously with BSF prepupae samples as positive control derived from a work conducted by Miguéns-Gómez et al. (2020), studying the enterohormone secretion ex vivo in human and pig intestine treated with in vitro digestions of three different protein sources.

5.2.2 Chemicals

All the chemicals used were of analytical grade. Sodium bicarbonate, α-Amylase (EC 3.2.1.1), Porcine Pepsin (EC 3.4.23.1), Porcine pancreatic lipase (EC 3.1.1.3), and Bile salts were provided by Sigma Aldrich, Co. (St Louis, Mo., USA). Pancreatin contains enzymatic components including trypsin, amylase, lipase, ribonuclease, and protease. The ELISA kit for total GLP-1 (catalog no. EZGLPT1-36K) was purchased from Millipore (Billerica, MA, USA). We used it according to the instructions provided by producers that could be easily obtained in the websites of each enterprise with detailed references. Gly-Pro-7- amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC) was obtained from Bachem AG (Bubendorf, Switzerland). Diprotin A (Ile-Pro-Ile) was supplied by Enzo Life Sciences International (New

York, USA. LDH kit was provided by QCA (Tarragona, Spain). All the other solvents, salts, acids, and bases were purchased from Sigma-Aldrich.

5.2.3 Simulated gastrointestinal digestion

The Black soldier fly prepupa samples were subjected to an *in vitro* gastrointestinal digestion, adapted from the INFOGEST harmonized protocol, as previously described Miguéns-Gómez et al. (2020). Briefly, the different prepupa samples were collected, the groups still whole finely ground at the moment, and mixed sequentially with three different simulated digestive fluids (salivary, gastric, and intestinal), each composed of a stock solution with a specific concentration of electrolytes, enzymes, $CaCl_2$ and water, in order to simulate the three corresponding typical phases of a whole digestion process. At first, to reproduce the oral phase, a different amount of each prepupa, specific to obtain a starting sample concentration of 0.12 g protein/mL simulated saliva, was directly solubilized in the salivary electrolytic stock solution in a ratio of 50/50 w/v. The mixtures were mixed for 2-5 min using an Ultra-Turrax T25 (IKA Werke, Staufen, Germany). Once these appeared homogeneous, the α-Amylase (EC 3.2.1.1) to achieve 75 U mL^{-1}, $CaCl_2$ (final concentration: 0.75 mM) and water were added. The mixture was adjusted at pH 7 and mixed again at 37°C for two minutes. After this, a specific volume of gastric electrolytic solution to reach a ratio of salivary mixture to gastric fluids of 50/50 v/v was added. To this second phase Porcine trypsin (EC 3.4.21.4) to achieve 2000 U mL^{-1}, $CaCl_2$ (final concentration: 0.075 mM) and water were added. After adjusting the pH to 3, the gastric phase was therefore maintained in agitation at 37°C for 120 min. For the last phase, the gastric phase volume was mixed with the same amount of simulated Intestinal fluid (50:50 v/v). Porcine pancreatic lipase (EC 3.1.1.3) to achieve 100 U mL^{-1}, Bile salt mixture (10 mM), $CaCl_2$ (final concentration: 0.3 mM) and water were added. The pH was further adjusted to 7 and the final solution was maintained in agitation at 37°C for other 120 min. Consequently, digested black soldier flies' samples were heated to 90°C for 30 min to stop the enzymatic reactions and centrifuged for 5 min, 4000 rpm and at 4°C, to separate the indigested part. Following this step, the supernatants were collected, lyophilized, and stored at -20°C until use. As

enzymes control, the same procedures were applied to an additional sample without inserting at the beginning of the process food to digest.

5.2.4 Characterization of Digestion Products by SDS gel electrophoresis

After the artificial digestion, a Bicinchoninic Acid (BCA) Assay for protein quantification of the digested, negative controls and enzyme control samples was carried out using a BCA kit (Pierce, Thermo Fisher Scientific) (Walker, J. M. 2009). Following, an SDS-PAGE was performed, to attest the successful digestion of the treated samples. The electrophoresis was carried out mixing 30 µl of each digested, undigested and enzyme control sample with a protein concentration of 2 µg/µL with 10 microliters of 4 × sample loading buffer (125 mM Tris HCl (pH 6.8), 2.5% (*w/v*) sodium dodecyl sulfate (SDS), 0.1% (*w/v*) bromophenol blue, 25% (*v/v*) glycerol, 25% (*v/v*) β-mercaptoethanol). After heated the mixture at 100 °C for 5 min, 15 µl from each sample were collected and loaded on a 16% polyacrylamide gel. A molecular weight marker (Page Ruler, Thermo Fisher Scientific) was included on each gel. Gels were then stained with colloidal Coomassie Blue (Bio-Rad Laboratories).

5.2.5 GLUTag Cell line culture

The GLUTag cells used in the present work were kindly donated by Prof. Staels (University Lille, Institut Pasteur de Lille, Lille, France) with permission of Prof. Drucker (Lunenfeld-Tanenbaum Research Insitute, Toronto, Canada). The medium where the cells were cultured was composed by 88% of DMEM (Dulbecco's modified Eagle's medium) containing 1g L−1 D-glucose, supplemented with 10% foetal bovine serum (Sigma-Aldrich, Madrid, Spain), 1% of 100U mL−1 / 100 mg L−1 Penicillin / Streptomycin and 1% of Glutamine (final concentration 2mM) (Lonza, O Porriño, Spain). The cells were incubated under a 5% CO2-humidified atmosphere at 37 °C. At least, three GLUTag cell passages were cultivated consequentially to allow each treatment to have an adequate number of biological replicates for the GLP-1 secretion test.

5.2.6 GLP-1 secretion test

GLUTag cells were plated onto 24-well plates precoated with Matrigel (Lonza, O Porriño, Spain) at a density of 200.000 cells/mL 24 h before the secretion study. Cells were then washed twice with PBS buffer and treated for 2h at 37 °C with the CP, PFR, PFP, Pagri samples following intestinal digestion dissolved in HEPES buffer (1.25 mM) at a concentration of 5 mg protein/mL. Following the same conditions of analysis, a previously digested sample of Buffalo worm was also tested with the BSF prepupae, in order to compare the GLP-1 stimulatory ability of our BSF samples with another insect species already tested. All the treatments were performed by duplicate in each cell plate and repeated for 3 passages. After the treatment the medium of each well was collected and stored at -80º C in aliquots of 25 µl until the determination of total GLP-1 and LDH release assay following the manufacturer's instructions. Then the cells were lysed with RIPA buffer and lysates were stored at -80º C, and then used to analyse total protein content by using a BCA kit (Pierce, Thermo Fisher Scientific) and for LDH quantification in the cells.

5.2.7 Determination of the DPP-IV inhibitory activity (% inhibition and IC_{50})

The potential inhibition of the black soldier fly samples before and after gastrointestinal digestion on DPP-IV activity was measured using 96-well microplates, according to the method used by Casanova-Martí, À., et al. (2019), with slight modifications (Casanova-Martí et al., 2019). The inhibition assay consisted in dissolving directly in the microplate 15 µL of DPP-IV enzyme (to reach 0.26 mU per well) and 10 µL sample containing 6 mg of proteins/mL per well (0.06 mg protein per sample) with 25 µL of 100 mM Tris·HCl assay buffer (pH 8.0). The microplate was pre-incubated for 10 min at 37 °C. Then, the assay was started by adding 50 µL of the chromogenic substrate H-Gly-Pro-AMC (final concentration 0.01 mM). The plate containing the mixtures was read in a microplate reader at Ex:380 nm/Em:460 nm at 37 °C each minute for 30 min. As reference inhibitor and positive control of the assay was used Diprotin A (Ile-Pro-Ile), well known to inhibit the DPP-IV enzyme. A blank sample (with no insect end enzyme) and an enzyme sample (enzymes with no insect) respectively as negative and enzyme control and a digested sample of Buffalo worm, to have a comparation with another insect species, were also tested. Each sample was tested

in triplicate. The DPP-IV inhibition activity of each sample was expressed as a percentage, derived from the difference between the enzyme activity in the presence of the test samples and the enzyme control sample's activity. Then, to calculate the IC_{50} (the concentration of the sample required to cause 50% inhibition of the enzyme activity), the DPP-IV inhibition experiment was performed again, using five different concentrations (ranging from 15 to 60 and from 3 to 0.5 mg of protein / mL respectively for the no digested and digested) of the previously tested control prepupa samples. The IC50 values, expressed by the estimated peptide concentration (μg mL^{-1}) and volume (μL), were determined by plotting the percentage of inhibition as a function of the test hydrolysate concentration using GraphPad prism v4.0 for windows.

5.2.8 Data analysis

The data are expressed as the mean ± SD or ± standard error and are the result of at least a triplicate analysis of independent samples. Differences between the means of the tested groups of samples were determined using one-way ANOVA test, followed by Tukey's post-hoc test. Statistics were performed using the SPSS software (SPSS, Chicago, USA). Only P-values < 0.05 were considered significant.

5.3 Results

5.3.1 Characterization of the samples

The content in lipids, proteins, ashes and other compounds, including chitin of BSF prepupae was evaluated to assess potential variations in the nutrients' distribution of the samples based on the different rearing or fermentation conditions. The complete proximate composition is reported in table 5.1.

Table 5.1. Nutritional composition of BSF prepupae. Values are expressed on dry matter basis and are the result of four replicate analysis. One-way ANOVA and Tukey's post-hoc test were used for multiple comparisons. Different letters (a,b,c) indicate significant differences (p-values < 0.05). *Caligiani et al (2018).

Composition (%)	**CP***	**PAgri**	**PFR**	**PFP**
Lipid (Soxhlet)	37.1 ± 0.1 [a]	29.9 ± 4.3 [b]	10.6 ± 0.3 [c]	16.4 ± 1.5 [c]
Proteins, from total AA (UPLC/ESI-MS)	32 ± 2 [a]	36.7 ± 0.3 [a]	36.2 ± 0.3 [a]	35.4 ± 0.6 [a]
Ash (Oven 550 °C)	19 ± 1 [a]	7.8 ± 2.3 [b]	6.5 ± 0.1 [b]	6.3 ± 0.1 [b]
Other compounds (including chitin)	11.9 ± 1 [a]	25.6 ± 0.7 [b]	46.7 ± 0.3 [c]	41.9 ± 0.6 [d]

How is possible to see from the results, the control prepupa has the highest percentage of lipids and ashes, which are instead present in significatively lower concentrations in the samples undergone to different rearing conditions and fermentation with the two different LAB strains. Because of the nature of the investigation presented in the following paragraphs, special attention shall be given to the protein content of the samples. As shown in table 5.1, it does not undergo any significant variation among the samples. Protein value in fact remain stable, ranging from 32.2 to 36.7 g / 100g of proteins. The significant higher amount in the content of other compounds in both PFR and PEP with respect to the other samples is essentially due to the sugar addiction to the insect substrates at the moment of LAB incubation to trigger the fermentative process.

5.3.2 Characterization of Digested Samples

To ensure that during the *in vitro* incubation of prepupae samples with digestive proteases were properly hydrolysed, digests were analysed by SDS–PAGE electrophoresis. Four samples were submitted to digestion, CP and Pagri, which differed in the diet in which the prepupae were reared, as well as two samples that had been previously fermented with

two different *lactobacillus* strains (PFR and PFP). The results are shown in figure 5.1, where digested and samples before digestion are analysed together. The SDS-PAGE analysis of the undigested CP and PAgri showed a very similar protein pattern, richest of bands also with a higher molecular mass. Equally, the PFR and PFP showed both the same profile but in this case presenting all the protein bands concentred in the 25-10 kDa range, probably as a result of the previous fermentation process. In any case, there was a clear difference between the protein pattern of the digested (+) and non-digested (−) samples. In the non-digested samples, we can see, in fact, the presence of protein bands which completely disappeared in the digested samples, showing that the digestive protocol, hydrolysing the proteins and generating small peptides not visible with this kind of analysis, degraded the initially present proteins. Even the previously fermented samples (PFR and PFP) showed fainter bands after simulated gastrointestinal digestion, suggesting a further degradation to smaller peptides.

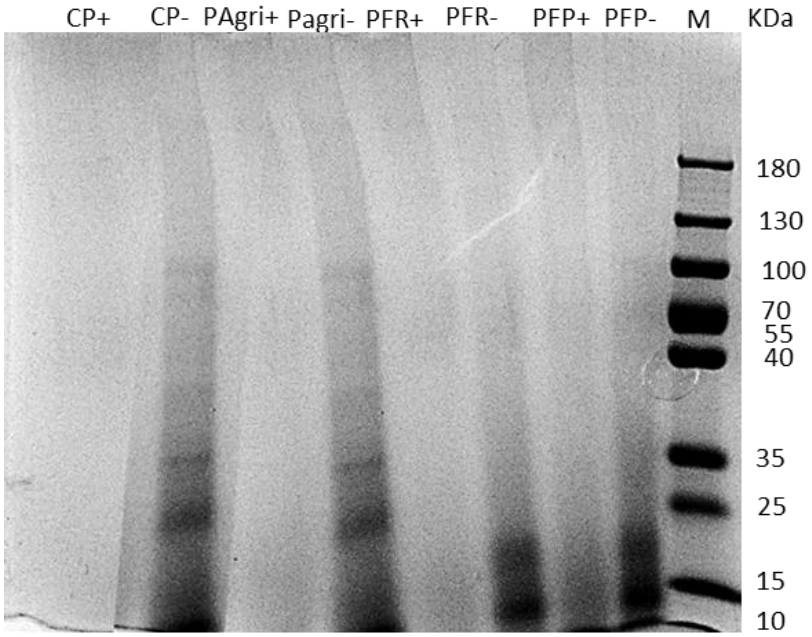

Figure 5.1 Protein hydrolysis after black soldier fly prepupae intestinal digestion. The digested sample are indicated with the mark "+", while the undigested controls are indicated with "–". The protein load was adjusted in each lane to 30 µg of protein. A molecular weight marker (10–180 kDa) was included. M, molecular weight marker.

5.3.3 Black soldier fly protein has DPP-IV inhibitory activity

The first purpose of this work was to assay whether BSF protein had DPP-IV inhibitory capacity, as well as to determine whether optimized rearing conditions or previous fermentation of the BSF would affect it. Figure 5.2a shows that BSF CP and PAgri undigested samples had a slight but significant DPP-IV inhibitory capacity. This was not different between the two samples. Surprisingly, on the contrary the fermentation of BSF with two different *Lactobacillus* strains did not increase but rather decreased the DPP-IV inhibitory activity of BSF. Then the effects of the same samples submitted to an *in vitro* gastrointestinal digestion was tested. The results are shown in figure 5.2b. It can be observed that the simulated intestinal digestion produced BSF hydrolysates with a very high inhibitory activity (more than 90%) against the DPP-IV enzyme. Such inhibition was greater than the response obtained from the digested Buffalo worm sample (51%) and even higher than the response of the positive control Diprotin A (CTR+) at the tested conditions (82% inhibition). A negative control using only the gastrointestinal digestion vehicle without insects sample was also carried out at the different concentrations used, showing no effects on DPP-IV activity (results not shown). As well as with the undigested samples, there were no significant differences between the BSF depending on the diet conditions. In this case, previously fermented samples followed by gastrointestinal digestion showed the same DPP-IV inhibitory activity than non-fermented samples. To better characterize the effects of simulated gastrointestinal digestion on DPP-IV activity of BSF protein, the IC50 was calculated for the CP protein before and after the digestion. Table 5.2 shows that the digested CP proteins showed a very low IC50 value, 10 times lower than the IC50 value of the undigested CP.

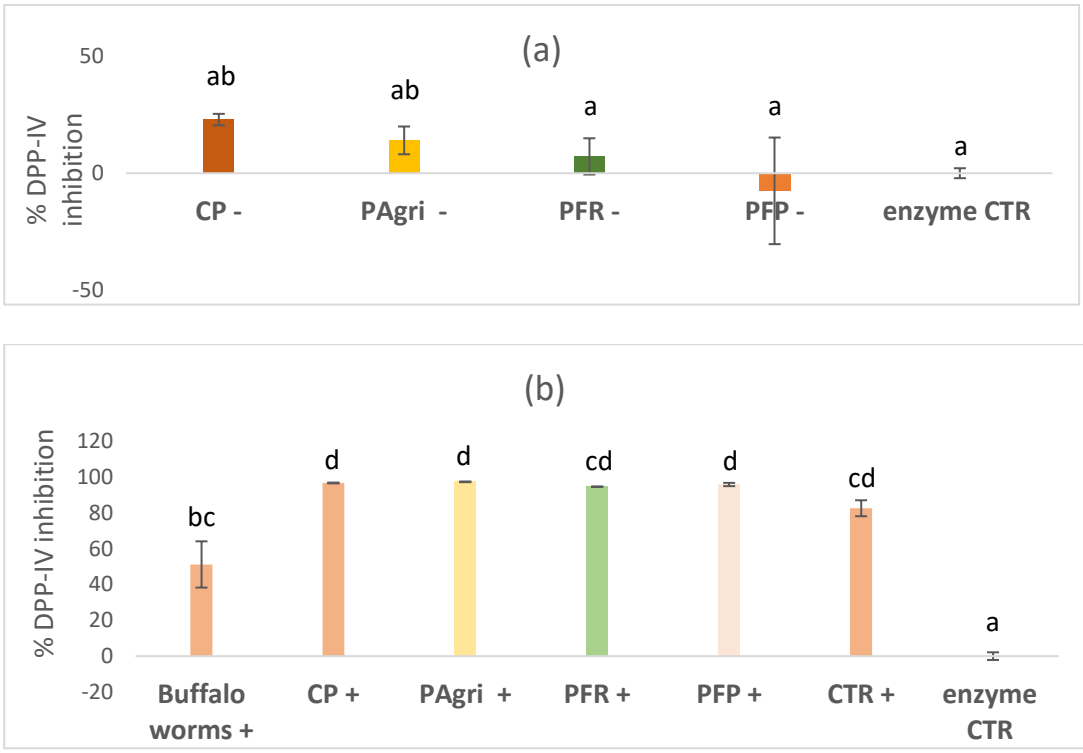

Figure 5.2 DPP-IV inhibitory activity of undigested (-) (a) and *in vitro* digested (+) (b) BSF prepupae compared with the enzyme control (enzyme CTR+), the inhibitory positive control (CTR+) and digested buffalo worms. The percent of DPP-IV inhibition was determinated using 6 mg of protein / mL as final assay concentration of sample. The results are expressed as the mean ± SEM of three replicates. One-way ANOVA and Tukey's post-hoc test were used for multiple comparisons. Different letters (a,b,c) indicate significant differences (p-values < 0.05).

Table 5.2 IC50 activity of control black soldier fly prepupa (CP) undigested (-) or submitted to a simulated gastrointestinal digestion (+). IC50 values are reported as the mean from triplicate assays ± SD.

Sample	IC50 (mg mL-1)
CP-	14.54 ± 0.11
CP+	0.14 ± 0.16

5.3.4 Intestinal digests of BSF prepupa stimulate GLP-1 secretion

Next the potentiality of BSF prepupae to activate at the intestinal level the GLP-1 secretion was evaluated *in vitro*, by using GLUTag cells. The enteroendocrine cell line was incubated

for one hour with 5mg protein/mL of the four different samples submitted to a simulated gastrointestinal digestion, to better mimic what might take place in the intestine.

First, we ruled out a potential cytotoxic effect of the treatments on the GLUTag cell line through LDH assay. The results obtained (table 5.3) showed that the percentage of LDH released to the medium was very low (< 3% in all cases) discarding toxic effects.

Table 5.3 LDH release to the medium after 1 hour digested BSF prepupa treatments in GLUTag cells. The % was determined using medium and cells from each treatment. The results are expressed as the mean ± SEM of six replicates from three different cell passages. One-way ANOVA and Tukey's post-hoc test were used for multiple comparisons. CP, control prepupa; Pagri, prepupa reared on an optimal agri-food by-products mixture; PFR, prepupa fermented with LAB rhamnosus; PFP, prepupa fermented with LAB plantarum.

	CP	PAgri	PFR	PFP	Vehicle-treated cells
% LDH released to the medium	2.7 ± 0.6	2.4 ± 0.6	2.6 ± 0.5	2.3 ± 0.1	1.2 ± 1.0

Figure 5.3 shows the total GLP-1 release to the medium after treatment with the different BSF samples. All the gastrointestinal digested samples led to a significant increase in the cell secretion of total GLP-1 compared to untreated cells. On the contrary, there are no statistical differences among the different BFS prepupae samples. Previous incubation of the prepupae with *Lactobacillus rhamnosus* or *L. plantatum* does not modify the GLP-1-secretory capacity of the gastrointestinally digested samples. Moreover, as is possible to see from the figure 5.3, the GLP-1 stimulatory ability found in all BSF prepupae is not significantly different from that obtained in the previously digested Buffalo worm sample.

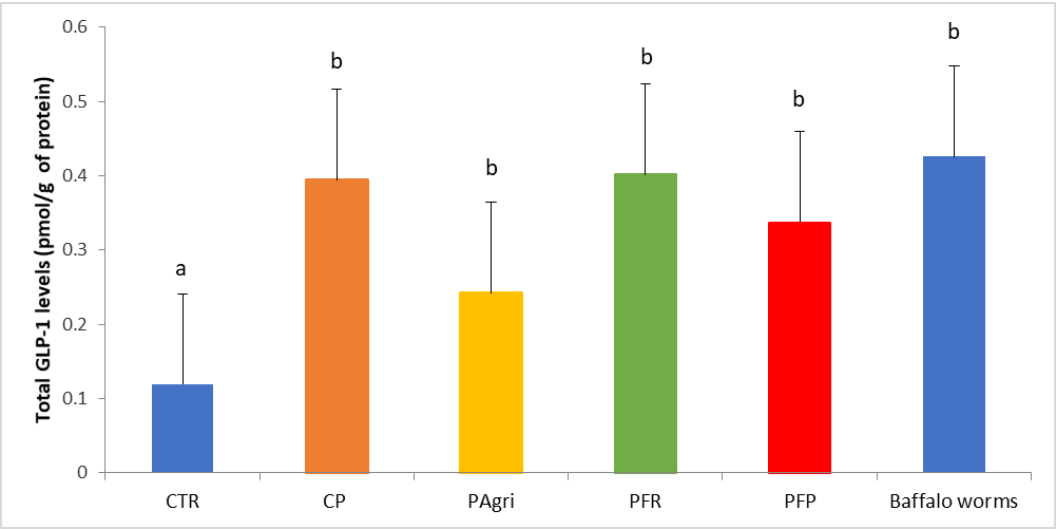

Figure 5.3. Effect of gastrointestinal digested prepupa samples on total GLP-1 secretion in GLUTag cells. Cells were incubated with 5 mg protein/mL of the different samples for 30 min at 37°C and total GLP-1 release to the medium was assessed. Values were normalyzed by protein content in each well. Results are presented as the mean ± SEM. The sample number was n = 6, from three different passages. One-way ANOVA and Tukey's post-hoc test were used for multiple comparisons. Different letters (a,b,) indicate significant differences (p-values < 0.05).

5.4 Discussion

The aim of the present investigation was to evaluate the intestinal response in humans after potential ingestion and the digestion of BSF prepupa samples, focusing on their potentialities at inhibiting the DPP-IV enzyme and stimulating GLP-1 hormone release. Furthermore, we aimed to compare whether this potential intestinal response was modulated by the diet in which the prepupae were reared, as well as to test if they could be improved by a previous fermentation with two different *Lactobacillus* strains.

The DPP-IV enzyme is strongly involved in regulating the glucose level in the bloodstream. Its main role is to rapidly inactivate the incretins (GLP-1), avoiding in a healthy subject a hypoglycemic effect (Arulmozhi & Portha, 2006). The increased incidence of human diseases such as diabetes, which on the contrary is a hyperglycemic condition, is leading to a constant search for new functional food able to prevent it, reducing the use of chemical formulations (Alkhatib et al., 2017). In this context, one of the most investigated aspects is

the ability of some protein sources to produce, after digestion, bioactive peptides with DPP-IV inhibitory activity. In this paper, we show for the first time that BSF proteins has DPP-IV inhibitory capacity. In order to test exactly the same biomass that our body comes in contact with, a gastrointestinal digestion was performed before testing the samples. Our results on DPPIV activity show that the digestion of the BSF led to a strongly increased inhibitory capacity. Even if there is no literature to refer on the bioactivity following an *in vitro* digestion of the BSF proteins, similar investigations related to other edible insects allowed to make some comparisons (F. Hall et al., 2018; Lacroix et al., 2019; Nongonierma et al., 2018; L. Yi et al., 2013). We also decided to test together with our samples a previously digested sample of another insect species, the Buffalo worm (*Alphitobius diaperinus)* to increase the chances of comparison. In this context, even if an DPP-IV inhibitory activity is present in all insect samples following gastrointestinal digestion, the BSF prepupae present a higher bioactive potential, suggesting higher enzyme inhibitory efficiency of the BSF compared to other species. However, our results on DPP-IV inhibitory activity can be considered in contrast with what was found on other works, in species as *Gryllodes sigillatus* or the same *Alphitobius diaperinus*. Here, in fact, the DPP-IV inhibitory activity after hydrolysis is in line with the response obtained testing our BSF prepupae sample (F. Hall et al., 2018; Lacroix et al., 2019). These differences in the inhibitory potential could be related to different hydrolysis conditions among the experiments. Anyway, in these works the hydrolysis step always leads to an increase DPP-IV inhibitory activity by all species of insects tested.

Despite different insect species and different hydrolysis conditions among the experiments, in fact, antidiabetic peptides production was observed mainly attribute to the work of specific enzymes, such as pepsin, known to cut proteins in appropriate positions to generate fragments exerting DPP-IV inhibitory activity (Lacroix et al., 2019). In fact, in works in which the bioactivity of edible insects as food sources on the intestine is tested, a proper enzymatic hydrolytic pre-treatment is necessary to obtain sufficiently high yields of peptides, main responsible for the most important biological activities (F. Hall et al., 2018; Sousa et al., 2020; Zielińska, Baraniak, et al., 2017). A slight inhibitory activity against the

enzyme DPP-IV has also been found in the undigested prepupa, especially in CP and PAgri samples. This can be explained by the high concentration of proteins within the insect biomasses and consequently by the presence of a potential endogenous enzymatic activity that can trigger even before the proteolytic treatments an initial break of insect proteins and a small production of bioactive peptides, generating inhibitory activity (Lacroix et al., 2019). Regarding the IC50 of these digests, no data on the specific BSF species to refer were as well available. If compared with that one found studying different food protein sources, the IC50 value of the BSF prepupa can be considered lower, suggesting a higher concentration in insects of inhibitory peptides. In the study of Casanova-Martí et al. (2019), for example, the IC50 value of chicken feet hydrolysates was around 30 mg estimated protein per mL. Even higher numbers were found by Nongonierma and FitzGerald (2015). Here the DPP-IV inhibitory potency of simulated gastrointestinal digested hemp, pea, rice and soy were evaluated and IC50 values found ranged from 1.85 ± 0.34 to 4.50 ± 0.55 mg dw hydrolysate mL^{-1}. Besides this, the IC50 found in our samples was found lower also respect the IC50 values observed for other insect hydrolysates. From *in vitro* digested *A. diaperinus* proteins, for instance, Lacroix et al. (2019) found a IC50 value around 1.0 mg mL^{-1} while Nongonierma, Lamoureux and FitzGerald (2018) reported a IC50 ranging from 0.40 to 1.0 mg mL^{-1} for crickets (Lacroix et al., 2019; Nongonierma et al., 2018). This actually makes the black soldier fly among the insect species investigated, one of the best candidates as alternative functional ingredient to the most common protein sources.

BSF prepupae digested protein was also tested for its ability to stimulate the enterohormone GLP-1 in an *in vitro* system. As known the GLP-1 is a kind of hormone belonging to the class of incretins and secreted from intestinal L-cell in response to meal ingestion. As mechanism of action this hormone stimulates the insulin and inhibit the glucagon, encouraging the blood sugar balance (Arulmozhi & Portha, 2006; Baggio & Drucker, 2007; Cui et al., 2020; F. Yi et al., 2013). The ability of some food to stimulate this hormone compared to others, gives them a particular interest as natural coadjutant to human health condition. Hence, numerous are the studies present in literature, whose aim is the study of how different food sources stimulate the system of incretins, using different

models (*in vivo, ex vivo, in vitro*). For example, in the study of Mochida et al. (2010) it is shown that meat hydrolysate has a high power in stimulating GLP-1 secretion. Even more, Geraedts et al. (2011) and W. L. Hall et al.(2003) found a good capacity in the GLP-1 hormone secretion in casein, whey, codfish, and eggs. Actually, a dual role at inhibiting DPP-IV and enhancing GLP-1 release *in vitro* has been shown for some food sources, including chicken feet, cuttlefish or whey proteins (Caron et al., 2016; Cudennec et al., 2015; Higuchi et al., 2013; Hutchison et al., 2015; Overduin et al., 2015). Although all interest in these potentialities, information lacks on how novel food sources, such us insects, could modulate the enterohormone release. The study of Miguéns-Gómez et al. (2020) is one of the few measuring the enterohormone secretion *ex vivo* in human/animal intestine treated with *in vitro* digestions of a specific insect and that gives information on the ability of these food source to stimulate GLP-1 secretion. Here is shown how the insect *Alphitobius diaperinus*, respect to other proteins sources, was the one of the most effective in inducing the secretion of total GLP-1 in human colon samples. As already explained in the materials and methods, we decided to test again this specific insect sample simultaneously with our BSF prepuapae, to cross check the responses and indirectly compare BSF species with other protein sources. The results obtained in the present study demonstrate the potential of Black soldier fly prepupae to increase GLP-1. In our investigation, the GLP-1 values obtained from the BSF prepupae treatments were actually the same compared to the other insect species tested here and studied in the work cited above. The GLP-1 response to the digested BSF prepupae suggests that this insect among the species currently investigated and compared to other protein sources presents a strong potential beneficial power in reduce glycemia.

Another goal of the present study was to investigate whether rearing the prepupae on different agri-food by-products respect to the control diet would modify the effects on the gastrointestinal tract of BSF. The specific by-products (40% minced tomato, 30% bran and 40% ground green beans) on which the PAgri was reared led to an improvement in terms of breeding performances (number of larvae and growth rate) respect the prepupae grown on the control diet (CP) (Barbi et al., 2020). Our results show that changes in the diet did

not lead to significant changes in the BSF properties, since results observed resembled those of the commercially fed insects. In fact, CP and PAgri showed similar DPP-IV inhibitory capacity, especially when gastrointestinally digested, as well as there were no differences in the levels of GLP-1 induced secretion by the digested samples. These results suggest that the protein and peptides contained in the prepupae were similar. This assumption is supported by the data obtained by proximate composition, which revealed that not significant changes were present in the protein content of the insects according to the diet administered. A similar condition has been observed by Fuso et al. (2021) where it is studied how different organic leftovers affected the protein fraction of BSF prepupae. Here it was shown how prepupa group grown on organic residuals principally composed of cereals and vegetable by-products, similar to those ones used for rearing our sample PAgri, was the only one that showed no significant differences in the protein content with respect to the control diet group, concluding that the different diets had identical effects (Fuso et al., 2021).

In the present study we also evaluated the effects of BSF fermentation using two strains of *lactobacillus*. Our results on the characterization of the digestion show that the fermentation process effectively broke down the BSF into smaller peptides since as expected, the undigested CP "-"and PAgri"-" presented more bands than the undigested fermented prepupae samples (PFR "-"; PEP"-"). Supposedly, in fact, the fermentation process leads to a pre-digestion of the food, affecting the protein fraction and starting to generate smaller peptides (Fadda et al., 2010; Stadnik & Keska, 2015). This results for the fermentation-treated and not digested samples in a higher concentration of bands in the part of the gel concerning proteins of minor size, ranging between 10 to 25 kDa. This result is completely in line with what resulted by other investigation, where the protein bands of fermented food samples were also observed to be in the same range (Kinariwala et al., 2020; Ni et al., 2018). It is known that microorganisms use proteins for their growth and metabolism. This explains why bacteria such as *Lactobacilli* during food fermentation perform proteolytic activity against substrate's protein fraction, promoting the release of increasingly small peptides, known to perform a wide range of biological activities,

including antidiabetic (Kinariwala et al., 2020). Although the high potential of *Lactobacillus* cultures for trigging a substrate's pre digestion during fermentation and producing bioactive peptides able to prevent hyperglycaemia it was already proven, in this study it was not possible to observe an improved DPP-IV inhibitory activity derived from incubation of BSF prepupa samples with selected LAB strains. When these microbial hydrolysates were subjected to simulated gastrointestinal digestion, they underwent even further cleavage, as observed in the electrophoresis gel. In this case, the DPP-IV inhibitory activity reached the same levels than the non-fermented BSF, suggesting that the final released peptides did not significantly differ. These results contrast with another study showing how a fermentative pre-treatment followed by an *in vitro* digestion instead leaded to a decreased antihyperglycemic activity respect to a hydrolysed non-fermented substrate (Jang & Kim, 2021). The reason for this difference is possibly related to a very low degree of proteolysis that characterized our undigested fermented samples, as shown in the electrophoretic gel. The small peptides generated by fermentation are very limited in number, not probably to lead to an effect on intestinal hormonal regulation. In this context, (Nongonierma et al., 2018) noted a decreased DPP-IV inhibitory activity following the simulated *in vitro* digestion of a fermented cricket protein. It was explained by the fact that the potential bioactive peptides already released by the bacterial pre-digestion could be broken down during the simulated digestion due the action of selected enzymes. In contrast, in our case the BSF digestion probably allowed the release of the peptides required for the DPP-IV inhibitory action, small peptides which were not released before by the fermentation only. Indeed, food bioactivity derived from a fermentation can depend on the microorganism species, growth substrate, time, and temperature of the environment etc. and this explain why the results observed in similar studies could be totally different (Torino et al., 2013). Both studies also agreed with the fact that gastrointestinal digestion might be considered when analysing the effects of protein and protein hydrolysates (Miguéns-Gómez et al., 2021).

Similar results were observed regarding GLP-1 secretion. In that case only the *in vitro* digested samples were tested, since previous literature suggest that digestion might modify GLP-1 secretory activity, either increasing or decreasing it, depending on the source

(Miguéns-Gómez et al., 2021). So as occurred with DPP-IV, previous fermentation of the BSF did not modify the GLP-1 stimulatory effect of the *in vitro* digested samples, suggesting that the digestion applied shortened to the same extent the fermented and non-fermented proteins and peptides, leaving to the release of bioactive peptides independent of previous fermentation.

5.5 Conclusions

In conclusion, the study conducted show a very high ability of digested insect proteins to inhibit the DPP-IV enzyme and to stimulate the GLP-1 secretion in GLUTag cells from all BSF prepupae. Our results highlight that the gastrointestinal digestion improves the DPP-IV inhibitory capacity of whole protein. Moreover, feeding the insects with a different composition diet does not modify the above-mentioned intestinal effects. We also showed that a previous fermentation with *lactobacillus* is not required for these activities, although we cannot rule out that different fermentation protocols could strengthen them. These results suggest that the BSF is one of the edible insects more suitable for human consumption. In addition to having an optimal nutritional profile, they seem able to exert beneficial activities on the human health. Being these preliminary tests conducted using *in vitro* conditions, more investigations are planned to understand if what found could be comparable to an *in vivo* model. At the best of our knowledge, this is the first time that black soldier fly insect is investigated as functional food for its hypothetical positive impact on human metabolic problems like diabetes. The good results derived from this experiment could be an incentive to deeper explore the beneficial effects on the human health from novel proteins sources, such as insects, still unexplored from numerous points of view.

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CHAPTER 6

Conclusions and future perspectives

Background scenario of the present PhD thesis

The world has been subject to continuous and periodic changes throughout its history, but during the last decades these changes underwent a significant acceleration. A huge increase in the world's population, followed by noticeable technological, economic and social improvements, resulted, on one hand, in a higher need of resources; on the other, in a much easier and direct access to them (Ehrlich et al., 2008). According to scholars, a much easier access to the supply, consumption and enjoyment of resources has led to enormous environmental negative effects, among which: 1: the increment of waste production; 2: higher environmental impact; 3: increased natural resources loss.

In this scenario, especially the agri-food sector was put under a considerable pressure due to the enormous increase in demand for agricultural goods and livestock which, in turn, led to an excess in the supply of such goods. The three key issues cited previously can thus be reformulated in this way: increase of agri-food by-products and leftovers, environmental damage derived from their inadequate disposal procedures and a fast and excessive consumption of clean water, soil for agriculture and livestock and food resources.

Without an immediate and concrete intervention, future generations will be condemned to suffer a lack of animal-based proteins, a lack of environmental resources, higher raw material costs, pollution, as well as a myriad of other important economic and social consequences. However, it is true that it is not the first time in history that the world faces crisis of food and natural resources. In the 1950s the need to feed poor countries pushed the use of strategies aimed at increasing the agricultural production yields. From then on, a massive and increasing use of fertilizers and pesticides revolutionized the world of agricultural production (Pimentel, 1996). Scholars use the term "green revolution" to describe such an event whereby the way of managing natural resources changes for development purposes. From that moment, the world has obviously made substantial progress, but to date, a green revolution is once again needed.

Scientific researchers are seeking sustainable ways to produce food, avoiding excesses in terms of requests, consumptions, and wastes deriving from a linear economic model, mitigating the future global food system crisis. In this view, edible insects have the potential

to become part of the solution (van Huis, 2015). They are able not only to grow and develop on a large range of organic waste, but also to transform these marginal biomasses into added value products, - such as fertilizers, biofuels, feed, pharmaceuticals - through the so-called process of “bioconversion”(Fowles & Nansen, 2019). Regarding the countless positive factors concerning the use of edible insects, we must highlight that their mass production would have a minimal environmental footprint with respect to other breeding systems and that their nutritional profile is rich in terms of saturated and unsaturated fatty acids, vitamins, minerals and high biological value proteins - which are often precursors of bioactive peptides (Jansson & Berggren, 2015; Shelomi, 2016; Rumpold & Schlüter, 2013).
Therefore, the use of edible insects could represent, on one hand, a solution to the issue of food waste preventing any environmental damage deriving from their disposal procedures; and, on the other, a new way to get secondary products to be re-used in a large range of industrial sectors, reducing the consumption of limited virgin resources. Moreover, the use of edible insects would satisfy the nutritional requirements of future individuals, compensating the future lack of the necessary protein sources (Varelas, 2019). Their use in the agri-food sector could change the way we handle resources, providing the food production system a new direction towards sustainability.
However, western countries are still in process of accepting insects or their derived products as part of a daily diet.
For this reason, many aspects related to their involvement in the circular economy system and their effects on human health are still unknown and have yet to be explored.
By now, just a few organic by-products have been extensively tested as a growth means for edible insects. Therefore, it is surely needed a much clearer analysis regarding the effects of agri-food leftovers on the insect's bioconversion ability, on species development, and on biomass resulting nutritional composition. Specific associations between agri-food waste or their combinations and insect species need to be defined in order to maximize bioconversion efficiency. In connection to insect rearing optimization, there will be the need to exploit insect extra biomasses and insect derived waste produced during bioconversion process, and scintific literature has not yet explored this topic. In addition,

the evaluation of insect biomass composition is hampered by the fact that the research scenario lacks accurate and fast methods concerning the quantification of some insect specific compounds as chitin.

Another knowledge gap concerns the ability of an insect-based diet to affect positively human health. Insects have the potential to produce bioactive peptides but, in the literature, there is a lack of information on the actual effects of their interaction with the human digestion system.

This PhD thesis will focus on all these aspects

Principal findings of this PhD thesis and research follow up

Getting animal feed and human food ingredients and products, from enriched biomasses is one aim of massive edible insect production. Using by-products and wastes derived from agri-food industries for the growth and development of insects makes this transformation process sustainable. In the past, promising results have already been observed by growing insects on food wastes. However, in the literature, there are scarce in-depth studies that evaluate the molecular profile of the different waste substrates and their effects on the growth and nutritional composition of the insect species. Greater knowledge on how to combine properly food waste with specific insect species to optimize the bioconversion process both in quantity and quality of the final products is required. In **Chapter 2** 49 different diets, each comprising different proportions of various agricultural by-products and split into three major groups based on the seasonal availability of substrates were carefully planned. Then, their specific nutritional composition and the effects of each diet on nutrients, especially on protein fractions of Black soldier fly prepupae was deepened. Black soldier fly prepupae biomasses presented a protein content ranging from 35% and 49% DM. The highest protein percentage was obtained when insects were fed with the Autumn group substrates, which are composed by pomace, legumes and corn A higher protein content was also observed in prepupae growing on protein rich substrates; this highlights how a certain quantity of vegetal proteins in the substrates is essential for a good transformation in insect proteins. However, it has been observed that, over a certain

quantity, the protein content of the substrate no longer leads to a protein increase of the insect biomass. Regarding the protein fraction quality, a higher quantity of essential amino acids was observed feeding prepupae again with the Autumn diets. Lysine, leucine and valine resulted to be the amino acids most affected by substrate's nutritional characteristics. One of the most important findings of this work is the observation that the essential amino acids profile of the prepupae, except very few exceptions, always perfectly satisfies the humans nutritional requirements suggested by FAO, indipendently from the diet used.

The research will demonstrate that the Black soldier fly prepupa is a perfect management tool of agricultural residues. Using agri-food residuals as growth substrate turned out to be highly acceptable in terms of both development and protein content of black soldier fly prepupae, that does not differ from the value achieved with a standard diet. When breeding a black soldier fly mass in order to produce feed and food, feeding insects with food residuals is possible, with the advantage of increasing the sustainability of the process and getting from a low value substrate a final biomass with a high protein quality. Besides all the positive results achieved by many experimental studies on food waste valorization by using insects, including Chapter 2 of this thesis, it was also here noted that when the starting nutritional value of agri-food substrates is poor, it might negatively affect the amount of enriched biomass and consequently on the protein yield which can be got by the bioconversion. In these terms, deeper investigation is suggested on the nutritional composition of the food by products and their combinations, based on their natural and commercial availability. This would allow create perfect combinations in which nutrients are balanced, meeting the nutritional requests of insects and optimizing the black soldier fly rearing in terms of maximum yield of protein. As a final note, it is known that almost all insect species naturally grow on a very large range of organic residuals found in nature. Thus, all insects are potentially efficient bio converters that could be investigated as means of enhancing of agricultural residues. It is therefore suggested to direct further research also towards species not yet investigated, in order to exploit the full potential of the insect rearing.

When analysing insect, and insect-derived food and feed ingredients, easy and effective analytical methods are essential for allowing a fast characterization of molecular composition of the insect biomass, in order to evaluate its quality and its future applicability in different sectors. Humidity, proteins, fats, ashes are the key elements which are commonly analysed in a biological matrix. The characterization procedure becomes trickier when the insect matrix is the object of the analysis, becouse of the nitrogen polysaccharide chitin in the insect body. Chitin is a polymer practically insoluble in most organic solvents, often complexed with other molecules, so it is difficult to quantify correctly it in a biological sample. In addition, its presence in organic matrices interferes with standard quantification methods for the determination of protein concentration. Thus, the search for faster and more precise chitin quantification methods is a very active research field. The best-known chitin quantification techniques are time-consuming, tricky, and complicated. They often required the fractionation of the matrix, to analyze directly the chitin fraction without the interference of the other compounds. Chromatographic analysis of free glucosamine following chitin acidic hydrolysis has recently appeared as one of the best methods, but still few applications are present in literature. The growing interest of the insect matrix, especially for food and feed purposes, requires that these biomasses are accurately characterized. In **Chapter 3** a new UPLC-ESI/MS method is presented that allows to quantify simultaneously chitin and proteins with a single method (never done before in literature) starting from the whole insect matrix insect, bypassing the problem of protein overestimation, and halving the analysis times. Before instrumental analysis, the insect matrix is to be subjected to acid hydrolysis according to the method already proposed for protein quantification of the same matrix (Caligiani et al., 2018). The applied time, temperature and acidity conditions lead to the simultaneous release of amino acids and glucosamine from respectively protein and chitin fraction, both chromatographically analysable after their derivatization with the AccQ Tag reagent. Validation tests for chitin quantification, i.e. linearity, LOD and LOQ, intraday and inter-day repeatability, and accuracy, were conducted on the Black Soldier Fly species. We confirmed hydrolysed commercial chitin and galactosamine as the best candidates to be used,

respectively, as external and internal standard for chitin determination. The method passed all the validation tests and proved to be faster, more precise, and simpler than compared to other independent methods for chitin only present in the literature. The chitin and protein content in Black Soldier Fly prepupae samples determined with the new method were found to be respectively 9,4 ± 0,8 % and 43,9 ± 0,7% on dry matter basis. We also tested the optimized method on another insect species (*Alphitobius diaperinus* larvae: 7 ± 1% chitin and 53 ± 1 % proteins), demonstrating its efficacy on the insect matrix in general, regardless of the species. Among the tests performed to assess the ability of the new method we analysed also two different commercial food supplements containing glucosamine and amino acids, to evaluate the applicability of the present validated technique also on different samples. The use of Glucosamine alone and/or in combination with other molecules (for example: hyaluronic acid) as ingredients of feed and food supplements is looking increasingly attractive for the proved positive effects on living beings' joint health (Hathcock & Shao, 2007). Encouraging, although still preliminary, results have been reported. Regarding the analysis of food supplement 1, the obtained values of 15% and 12% respectively for glucosamine and proteins perfectly matched those reported on the label. For the food supplement 2 instead, we did not get the precise values, but from the results, a majority of amino acids in the substrate compared to glucosamine was observed, as we expected it according to the chemical formulation declared in its package.

In this perspective, further tests are recommended. The procedure may require some customization depending on the form the analyte that is present in these dietary preparations, that could differ from each other and from that one in the insect sample. In these terms, it would be possible to use the method for controlling that the values present in the pharmaceutical formulations coincide with those showed on the label, acquiring a very high value as a tool of product safety control.

Therefore, although this method has been developed and optimised for use on the insect matrix, as future perspective, we could extend it on a wide range of matrices, where direct and accurate determination of chitin and/or protein content is required.

As discussed in Chapter 2, Black Soldier Fly is a promising means of bio-waste's valorization through bio-conversion processes of residual biomasses into bio-fertilizers, biodiesel, feed ingredients etc. In a global context in which the interest in waste materials management and enrichment is growing considerably, it is also of importance to find a way to valorize also the BSF derived wastes generated during the development of the insects on agri-food residues and based on their resulting composition, finding for them useful applications. In **Chapter 4** we performed a solid-state fermentation with 2 lactic acid bacteria (LAB) strains, isolated from food, as alternative valorization instrument, on BSF prepupae biomass and BSF-derived wastes (pupal membranes and adult insects at the end-of-life cycle) produced during bioconversion processes. In particular, a study of the difference in the molecular composition between fermented and unfermented above insect-derived waste materials, focusing on lipid, protein, and chitin fraction was carried out, in order to evaluate positive or negative variations in the resulting biomass quality following fermentation. The chapter explored the fermentation for its ability to enhance also insect-derived wastes, in a future perspective of having a "zero waste" insect bioconversion process. The bulk evaluation of fat, moisture, ashes composition of insect-derived biomasses was carried out by using standard procedures while protein and chitin content were evaluated by using the method studied and validated in Chapter 3, overcoming the problem of analyzing the two parameters separately. Fatty acid profiles were determined by GC-MS and the formation of peptides or chitin oligomers was carried out by LC/ESI-MS methodologies. A metabolomics approach by 1H NMR was also applied to get further insight in lipid insect fraction. The lipid and protein fractions were found the most affected by the fermentation: the fermented biomass had a different fatty acid and amino acids profile as compared to unfermented mass. The production of few small peptides was also observed, but we observed no chitin oligomers formation after the fermentation process. The results got in this study lead to this conclusion that, by exploiting the typical metabolic behaviour of fermenting bacteria, it is possible to transform insect wastes, until now unutilized, in valued resources. For the future use of insect waste materials in the industrial field, we encourage further studied in order to better understand possible functional properties of insect

fermented biomasses. Thanks to the improved nutritional composition, including fermented prepupae in a balanced diet as feed or food ingredient is an option (Castro-López et al., 2020; Kewuyemi et al., 2020; Shuping et al., 2012). For this aim the effects of these products on living organisms should be carefully investigated. Some tests on the antimicrobial activity of these fermented samples have already been carried out and promising positive results were obtained (Hadj Saadoun et al., 2020). The possibility of obtaining natural antimicrobials from these unused insect biomasses, to be used for livestock breeding, would be an excellent way to control the negative impact on human health derived from antibiotic residuals in animal-based food (Barton, 2000). Antibiotics in facts are general added in animal feed to increase production, protecting from infections. Insect wastes after fermentation could also be used as natural fertilizers, reducing dependence on chemical soil improvers (Mengqi et al., 2021). It is known that the fermentation can improve the degradability of molecular fractions and the availability of nutrients in waste material (Varelas, 2019).

Besides sustainability, edible insects can then be seen as the best alternative to animal proteins for future human consumption, thanks to a promising nutritional composition and, in particular, the high quality of the protein fraction. The biological activities recently showed by protein hydrolysates from edible insects, including antioxidant, antihypertensive, and anti-inflammatory make insects a promising tool also in protecting human health status (Bulet et al., 1999; Chernysh et al., 2002; de Castro et al., 2018; Zielińska et al., 2017). Among all bio properties ideally deriving from insect protein fraction, particular attention is placed on antihyperglycemic potential. The global increased incidence of diabetes is leading to a constant search for new functional food able to prevent it, reducing the use of chemical formulations (Alkhatib et al., 2017). An anti-diabetic potency *in vitro* by insect bioactive peptides was attributed to some species (*Tenebrio molitor, the Gryllodes sigillatus* and *Alphitobius diaperinus*) (Dávalos Terán et al., 2020; Lacroix et al., 2019; Nongonierma et al., 2018; Rivero-Pino et al., 2021). However, a lack of information for insect in general and black soldier fly in particular, is present, (Bessa et al., 2020; Wang & Shelomi, 2017). Thus, more *in vitro*, ex vivo and *in vivo* interaction studies

on insect regulatory ability of the glycaemic response in humans should be performed, in order to make the health benefits of insect-based diet clearer. Hence, in **Chapter 5** modulatory activity of hydrolysed black soldier fly prepupae by simulated gastrointestinal digestion on the enterohormone GLP-1 and on its natural inhibitor, DPP-IV, respectively a hormone and an enzyme involved in human glycemic response, was examined. With the aim of studying whether valorization actions such as optimized growth substrates or prior fermentation could affect positively human response, black soldier fly prepupae reared on a selected combination of agri-food by products and black soldier fly prepupae derived from the study conducted in chapter 4, i.e., fermented with two different LAB strains, were tested. GLP-1 secretion *in vitro* in human GLUTag Cell line treated with 5mg protein/mL of in digestions of black soldier fly prepupae samples was measured. All the black soldier fly prepupae proven very efficient in inducing the secretion of GLP-1. Also, the DPP-IV inhibitory capacity of digested black soldier fly proteins was studied. A very high inhibitory activity against the DPP-IV enzyme was obtained also in this case from all the insect hydrolysates, with a % inhibition always greater than 80% and IC50 (sample concentration to cause 50% inhibition of the enzyme activity) of 0.14 mg/ mL. With this investigation now, it is possible to confirm that an antidiabetic effect can be generated from black soldier fly proteins after *in vitro* gastrointestinal digestion. Moreover, optimized diets or fermentation processes preceding the digestion did not negatively or positively affect the efficacy of the answer. The results obtained are very promising. Besides the optimal nutritional profile that already makes it one of the edible insects more suitable for human consumption, black soldier fly species have many chances in future to be considered functional food. Being, at the best of our knowledge, the first time that black soldier fly is studied for its ability in improving glycemic regulation, deeper investigations are suggested to confirm the results obtained. It is also advisable to consider other variables in addition to those used, such as other diets formulations. The antidiabetic effect is a promising bioactivity deriving from protein fraction of black soldier fly biomass, nevertheless other beneficial biological potentials on the human health are expected deriving from this species. More extensive studies are suggested with the aim to test other biofunctional proteins coming from the

black soldier fly protein fraction, which could increase the value of the insect-based food product. Finally, the results in this investigation were obtained by using an *in vitro* intestinal model. The effect *in vivo* could be different. Real digestion conditions, the bioavailability of black soldier fly derived peptides, the intestinal portion where they are absorbed, and the proximity with the cells that release incretins are all factors which could affect differently the antidiabetic potential of the insect base product and should be properly studied. The promising results obtained in this PhD thesis with an *in vitro* model suggest also exploring the effect *in vivo*. Results showing the ability to prevent health risks could contribute to increasing public awareness and acceptance concerning insect consumption.

Future Perspectives

Thanks to the data in this thesis, black soldier fly has been further confirmed as a way to obtain biomass enriched in high-quality protein and an excellent tool for different agri-food waste management, alleviating the environmental risk. It is a species that can be used in bioconversion processes, thank his ability to grow efficiently on a large range of food by-products. The wastes generated from this process, mainly linked to the life cycle of the insect, can be nutritionally enriched with known valorization techniques, such as LAB fermentation. Hence, also insect waste material can be managed and transformed into high-value products ideal to be used as enriched feed ingredients or bio fertilizers. To find the right application, it will be essential to know the molecular composition of the insect products and assess the risks and benefits they may bring if used with specific aims. Knowing the right chitin content of the biomass in this case is essential, because of its known anti-nutritional effects. As a result, characterizing this enriched biomass efficiently and accurately will be a fundamental point, which can be considered definitely improved by the results of this thesis. Besides this, insects are considered for their optimal nutritional composition the human food of the future. Having found an *in vitro* biological activity, i.e. antihyperglycemic effect, exerted from bioactive peptides released after simulated gastrointestinal digestion, makes black soldier fly a species even more appropriate for food purposes.

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Supplementary Material

Table S 4.1: Complete amino acid composition (g AA/ 100g AA) of BSF adults, puparia and prepupae unfermented and fermented with L. rhamnosus (1473) and L. plantarum (285). *Significance (<0.05) of each fermented sample respect its corresponding unfermented material (T-Test comparation).

AA	*AD_Unf*	*AD_1473*	*AD_285*	*PUP_Unf*	*PUP_1473*	*PUP_285*	*PRE_Unf*	*PRE_1473*	*PRE_285*
ala	5.54 ± 0.25	5.41 ± 0.19*	5.14 ± 0.21*	4.32 ± 0.61	3.59 ± 0.09*	3.91 ± 0.32*	4.18 ± 0.15	4.54 ± 0.22*	3.91 ± 0.32*
asp	7.33 ± 0.01	6.50 ± 0.55*	6.49 ± 0.34*	2.51 ± 0.42	1.21 ± 0.15*	1.45 ± 0.17*	5.67 ± 0.69	7.28 ± 0.14*	6.60 ± 0.57
arg	4.70 ± 0.83	4.32 ± 0.27*	4.52 ± 0.32*	2.95 ± 0.75	3.19 ± 0.23	2.50 ± 0.15	3.52 ± 0.88	4.08 ± 0.12	3.59 ± 0.18
gly	3.89 ± 0.32	3.66 ± 0.29*	3.69 ± 0.13*	5.15 ± 0.15	4.82 ± 0.23*	4.78 ± 0.37*	4.09 ± 0.15	4.22 ± 0.08*	3.99 ± 0.18*
his	0.68 ± 0.07	3.64 ± 0.21*	3.80 ± 0.20*	1.78 ± 0.05	3.50 ± 0.30*	2.78 ± 0.17	2.96 ± 0.34	3.49 ± 0.05*	3.09 ± 0.26*
ile	3.55 ± 0.12	3.36 ± 0.21*	3.36 ± 0.04*	1.79 ± 0.05	1.77 ± 0.01*	1.64 ± 0.11*	2.67 ± 0.03	3.17 ± 0.10*	2.88 ± 0.16*
leu	5.69 ± 0.20	5.10 ± 0.17*	5.29 ± 0.21*	3.45 ± 0.12	3.24 ± 0.07*	3.23 ± 0.23*	4.62 ± 0.11	5.17 ± 0.12*	4.70 ± 0.23*
met	1.77 ± 0.08	2.11 ± 0.12	2.34 ± 0.09	0.71 ± 0.01	1.08 ± 0.07*	0.82 ± 0.06	1.28 ± 0.01	1.92 ± 0.02*	1.82 ± 0.08
phe	3.75 ± 0.33	3.99 ± 0.12*	4.28 ± 0.22	1.99 ± 0.03	2.75 ± 0.18	2.17 ± 0.13	3.24 ± 0.11	3.99 ± 0.03*	3.92 ± 0.18*
pro	3.45 ± 0.06	3.64 ± 0.41	3.43 ± 0.07*	3.51 ± 0.10	3.42 ± 0.09*	3.58 ± 0.24*	3.68 ± 0.02	4.01 ± 0.13*	3.57 ± 0.24*
ser	3.33 ± 0.32	3.46 ± 0.31	3.31 ± 0.11*	2.57 ± 0.09	2.78 ± 0.06*	2.75 ± 0.18	3.05 ± 0.14	3.31 ± 0.11*	3.29 ± 0.13*
thr	3.71 ± 0.12	3.41 ± 0.20*	3.49 ± 0.16*	1.70 ± 0.04	1.80 ± 0.08	1.77 ± 0.12*	2.88 ± 0.07	3.21 ± 0.11*	3.16 ± 0.10*
val	4.74 ± 0.06	4.13 ± 0.13*	4.32 ± 0.10*	3.94 ± 0.22	3.59 ± 0.06*	3.50 ± 0.23*	4.29 ± 0.13	4.57 ± 0.09*	4.02 ± 0.22*
lys	2.99 ± 0.08	2.02 ± 0.33*	2.19 ± 0.22*	0.87 ± 0.02	0.17 ± 0.12*	0.14 ± 0.06*	3.02 ± 0.64	2.68 ± 0.25*	2.42 ± 0.41*
tyr	3.98 ± 0.40	4.13 ± 0.11*	4.62 ± 0.26	3.60 ± 0.16	3.96 ± 0.14	3.42 ± 0.18*	4.52 ± 0.29	4.88 ± 0.11*	5.02 ± 0.27*

glu	9.01	±	0.38	8.83	±	0.62*	8.23	±	0.37*	3.90	±	0.63	2.21	±	0.22*	2.44	±	0.30*	5.65	±	0.57	8.74	±	0.12*	8.19	±	0.95
cys	0.47	±	0.01	1.43	±	0.20*	1.74	±	0.18*	0.45	±	0.02	1.29	±	0.05*	1.08	±	0.16*	1.90	±	0.10	1.40	±	0.05*	1.45	±	0.18*
trp	0.96	±	0.07	0.74	±	0.05*	0.79	±	0.05*	1.37	±	0.08	0.90	±	0.04*	0.96	±	0.10*	2.60	±	0.20	0.98	±	0.02*	0.92	±	0.01*

Table S 4.2: Peptides identified in protein extracts of BSF adults (a), puparia (b) and prepupae (c) unfermented and fermented with L. rhamnosus and L. plantatum by UPLC-ESI/MS analysis. The table reports information about the retention time (RT) of the peptide peaks and mass-to-charge ratio of characteristic ions of each peak, and the peptide abundance (semiquantitative %).

a)

RT	m/z	AD_Unf	AD_1473	AD_285	RT	m/z	AD_Unf	AD_1473	AD_285	RT	m/z	AD_Unf	AD_1473	AD_285
15.6	146	0.00145	0.00216	0.00202	47.4	272	0.00016	0.00042	0.0005	20.1	393	0.00026	0.00084	0.00086
43	147	0.00022	0.00183	0.00135	20.4	277	0.00021	0.00061	0.0006	13.7	397	0.00005	0.00044	0.00052
55.6	149	0.00243	0.00606	0.00703	23.5	279	0.00021	0.00056	0.00059	13.2	409	0	0.00056	0.00059
14.8	151	0.00045	0.00139	0.00184	28.9	287	0.00006	0.00023	0.00031	23.6	409	0.00027	0.00083	0.00082
38.4	158	0.00134	0.00547	0.00586	20.4	295	0.00019	0.00065	0.00069	27.2	411	0.00021	0.00065	0.0006
14.2	162	0.00066	0.00121	0.00111	23.5	301	0.0003	0.00083	0.00098	21.2	415	0.0002	0.00056	0.00065
12.2	169	0.00041	0.00088	0.0009	17.5	305	0.00029	0.00154	0.0015	22.7	423	0.00044	0.00137	0.00132
41.5	177	0.00019	0.00061	0.00065	12.3	310	0.00007	0.00768	0.0074	27.1	428	0.00048	0.00152	0.00151
15.8	179	0.00016	0.00125	0.00115	11.8	315	0.00002	0.00066	0.00058	21.2	432	0.00019	0.00066	0.00073
10.9	181	0.00034	0.0044	0.00427	31.7	318	0.00023	0.00079	0.00065	21.2	437	0.00021	0.00069	0.00069
26.4	183	0.0008	0.00247	0.00232	46.5	318	0.00036	0.00152	0.00118	23.6	453	0.00026	0.00075	0.00073
15.4	188	0.00471	0.01111	0.00648	21.6	321	0.00046	0.0013	0.00147	22.3	454	0.00016	0.00048	0.00045
20.4	189	0.00013	0.00049	0.0007	24.9	323	0.00029	0.00086	0.00086	18.3	460	0.00005	0.00031	0.00045
16.5	190	0.00022	0.00177	0.00158	18.9	327	0.0003	0.00091	0.00101	28.1	472	0.00052	0.00154	0.00144
14.6	195	0.00023	0.00076	0.00065	20.4	335	0.0005	0.0017	0.00163	23.6	475	0.00029	0.00092	0.00084
55.8	195	0.00031	0.00254	0.0022	19.2	340	0.00047	0.00134	0.00144	22.3	476	0.00039	0.00104	0.00102
15.2	196	0.00075	0.00223	0.0023	45.1	343	0.00259	0.00829	0.00907	22.3	481	0.00016	0.00058	0.00054
12	199	0.00013	0.00186	0.00166	24.9	345	0.00032	0.00104	0.00107	23.6	490	0.00007	0.00025	0.0002
54.3	204	0.00058	0.0019	0.00187	18.9	349	0.00026	0.00084	0.00097	34.8	492	0.00018	0.00044	0.00048
37.7	212	0.0002	0.00062	0.00078	35.4	361	0.00015	0.00079	0.00049	24.5	506	0.00026	0.00079	0.00086
48.2	219	0.00012	0.00049	0.00041	19.2	362	0.00028	0.00095	0.00092	28.9	516	0.00046	0.00143	0.00144
13.4	220	0.00044	0.00191	0.00186	22.7	365	0.00042	0.0013	0.00119	23.2	520	0.0002	0.00055	0.00048
36.9	226	0.0002	0.00074	0.00071	26.1	367	0.00033	0.00092	0.00091	25.3	550	0.00021	0.00061	0.00074
50.1	228	0.00528	0.01885	0.02	20.1	371	0.00029	0.00085	0.00086	29.6	560	0.00032	0.00098	0.00111
50.7	230	0.00127	0.00406	0.00448	52.2	371	0.0015	0.00494	0.00529	24	564	0.00012	0.00047	0.00039
40.4	244	0	0.00065	0.00046	21.6	379	0.00049	0.00162	0.00158	26	594	0.00012	0.00048	0.00052
23.6	245	0.00009	0.00038	0.00038	14.5	384	0.0003	0.00049	0.00045	30.2	604	0.00025	0.00063	0.00065
12.5	254	0.00048	0.00096	0.00095	26.1	384	0.00035	0.00102	0.00104	30.8	648	0.00014	0.00035	0.00038
11.9	268	0.00015	0.0008	0.00081	25.7	387	0.00032	0.00071	0.00062					

b)

RT	m/z	PUP_Unf	PUP_1473	PUP_285		RT	m/z	PUP_Unf	PUP_1473	PUP_285		RT	m/z	PUP_Unf	PUP_1473	PUP_285
15.2	113	0.002	0.00986	0.01073		13.9	247	0	0.00019	0		26	384	0.00027	0.00103	0.00093
12.9	128	0.00035	0.00185	0.00142		12.5	254	0.00028	0.0005	0.00044		23.6	387	0.00012	0.00078	0.00056
19.9	128	0.00002	0.00081	0.0002		21.8	257	0.00011	0.00045	0.00044		25.7	387	0.00015	0.00101	0.00073
15.2	141	0.0005	0.00253	0.00287		23.7	266	0.00005	0.00033	0.00014		20.1	393	0.0002	0.00069	0.00072
13.2	143	0.0001	0.00062	0.00038		47.3	272	0.00013	0.00045	0.00041		14.3	407	0.00004	0.00036	0.00001
15.7	146	0.00063	0.00495	0.00319		20.4	277	0.00016	0.00063	0.0003		23.6	409	0.00019	0.00088	0.00088
55.6	149	0.00196	0.00696	0.00672		23.5	279	0.00013	0.00055	0.00054		27.1	411	0.00016	0.00054	0.00053
38.3	158	0.00114	0.00408	0.00294		17.7	281	0.00003	0.00022	0.00003		17.7	413	0.00003	0.00017	0.00002
16.4	162	0.00021	0.00165	0.00126		17.4	283	0.00017	0.00059	0.00056		21.2	415	0.00014	0.00051	0.00047
14.7	163	0.00006	0.00061	0.00043		29	287	0.00006	0.00023	0.0002		22.6	423	0.00035	0.00125	0.00111
10.5	166	0.00172	0.00836	0.00055		20.4	295	0.00015	0.00062	0.00056		27.1	428	0.00036	0.00129	0.0013
12.8	168	0.00015	0.00074	0.00056		32.3	299	0.00006	0.00019	0.00026		21.2	432	0.00016	0.00057	0.00049
12.9	170	0.00003	0.00061	0.0002		23.5	301	0.00023	0.00084	0.00079		21.2	437	0.00016	0.00054	0.0005
41.5	177	0.00019	0.00062	0.00061		14.7	304	0	0.00076	0		23.6	453	0.00021	0.00073	0.00075
15.2	181	0.00088	0.00407	0.0049		17.4	305	0.00022	0.00073	0.00077		22.3	454	0.00011	0.00047	0.00041
26.3	183	0.00068	0.00252	0.00211		27.5	309	0.00007	0.00056	0.00056		13.8	455	0	0.00013	0
15.5	188	0.00431	0.0138	0.00042		12.3	310	0.00012	0.00344	0.00187		23.6	462	0.00019	0.00066	0.00068
20.4	189	0.00012	0.00115	0.00042		21.6	321	0.00003	0.00139	0.00131		36.1	463	0.00001	0.00028	0.0001
16.6	190	0.00019	0.00064	0.00065		24.9	323	0.00024	0.00076	0.00072		23.6	467	0.00023	0.00086	0.00081
23.6	192	0.00016	0.00112	0.00064		18.8	327	0.00023	0.00091	0.00078		28	472	0.00035	0.00138	0.0013
14.6	195	0.0001	0.00036	0.00029		23.6	328	0.00004	0.00027	0.00017		23.6	475	0.00024	0.00082	0.00086
55.4	195	0.00047	0.00144	0.0015		20.4	335	0.00028	0.00123	0.00138		22.2	476	0.00025	0.00089	0.00084
15.3	196	0.00033	0.00312	0.00261		16.9	338	0	0.00176	0.00003		22.2	481	0.00011	0.00045	0.00041
12	199	0	0.00024	0.00018		19.2	340	0.00039	0.00153	0.00138		34.8	492	0.00012	0.00038	0.00038
15.5	205	0.00098	0.00303	0.00009		45.1	343	0.00214	0.00767	0.00745		28.8	516	0.00035	0.00123	0.00123
37.6	212	0.00014	0.00047	0.00044		24.9	345	0.00023	0.00096	0.00087		23.1	520	0.00013	0.00042	0.00044
16.1	215	0	0.00026	0.0002		18.8	349	0.00021	0.00077	0.00079		25.2	550	0.00016	0.00058	0.00058
18.3	217	0.00005	0.00044	0.00011		36.1	350	0.00002	0.00047	0.00024		29.5	560	0.00025	0.00086	0.00086
48.2	219	0.00015	0.0004	0.00036		12.6	354	0	0.00048	0.00022		24	564	0.00009	0.00033	0.00032
13.5	220	0.00017	0.00066	0.00051		15.2	355	0.00074	0.00363	0.00417		25.9	594	0.00011	0.0005	0.00035
23.7	226	0.00012	0.00099	0.00047		38.7	357	0.00005	0.00054	0.00026		30.1	605	0.00011	0.00046	0.00034
36.8	226	0.00018	0.00066	0.00066		19.2	362	0.00023	0.00083	0.00078		36.1	625	0.00002	0.00036	0.00014
13.5	227	0.00011	0.00056	0.00046		22.6	365	0.0003	0.00112	0.0011		26.9	634	0	0.0006	0
50.1	228	0.00504	0.01888	0.01756		26.1	367	0.00023	0.00082	0.00079		38.7	640	0.00002	0.00037	0.00015
50.7	230	0.00113	0.00448	0.0042		20.1	371	0.00022	0.00075	0.00079		30.7	648	0.00008	0.0003	0.00025
26.9	231	0	0.00115	0		52.1	371	0.00122	0.00445	0.00386		36.1	681	0	0.00012	0.00002
14.6	238	0	0.00023	0.0002		38	377	0.0001	0.00134	0.00063		31.3	693	0.00003	0.00011	0.00011
45.1	240	0.00153	0.00577	0.00541		21.6	379	0.00042	0.00154	0.00133		42.4	715	0.00002	0.00054	0.00022

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c)

RT	m/z	PRE_Unf	PRE_1473	PRE_285	RT	m/z	PRE_Unf	PRE_1473	PRE_285	RT	m/z	PRE_Unf	PRE_1473	PRE_285	RT	m/z	PRE_Unf	PRE_1473	PRE_285
13.2	105	0.00266	0.00009	0.00101	38.4	266	0.00544	0	0	25.7	430	0.00025	0.00021	0.00009	27.1	656	0	0.001	0
12.6	116	0.00957	0.001	0.00197	47.3	272	0.00046	0.00033	0.00048	11.8	431	0	0.00014	0.00012	29.6	656			0.00042
16.4	116	0.00849	0	0.00105	12	274	0.00045	0.00009	0.00011	21.2	432	0.00075	0.00051	0.00079	25.8	670	0.00034	0.00035	0.00005
10.5	120	0.16075	0.00964	0.04163	20.4	277	0.00081	0.00054	0.00059	21.2	437	0.00082	0.0005	0.00075	27.3	672	0.00303	0	0
12.8	128	0.00065	0.00024	0.00259	22.3	279	0.00045	0.00051	0.0006	19.4	438	0.00035	0	0	28.2	672	0.00159	0.00051	0.00005
10.1	129	0	0.00068	0.00107	12	280	0	0.00034	0.0003	15.8	448	0.00102	0.00014	0.00003	30	672	0.00151	0.00067	0
17.1	130	0.01346	0.00927	0.01404	17.4	283	0.00084	0.00054	0.00067	22.3	453	0.00097	0.00059	0.00016	24.9	673	0.00051	0	0.00015
12.5	132	0.00192	0	0.00014	20.8	287	0.00021	0.00016	0.00015	23.6	453	0.00098	0.00061	0.00079	25.2	674	0.00067	0	0
13.2	132	0.0038	0.00029	0.00052	20.3	288	0.00065	0.00003	0.00003	24.5	453	0.0005	0.00031	0.00046	26	675	0.00173	0	0
26	136	0.00075	0.00076	0.00005	19	291	0.00084	0.00049	0.00072	28	455	0.00057	0.00035	0.00052	20.2	676	0.00047	0	0
12.5	143	0.0047	0	0.00021	19.7	294	0.00117	0	0	21.2	460	0.00019	0.00014	0.00014	25.8	683	0.00016	0.00017	0
13.2	143	0.00412	0.00277	0.00302	46.2	294	0.00241	0	0	23.6	462	0.00086	0.00062	0.00092	24.9	687	0.00015	0.00056	0.00008
13.2	143	0.00412	0.00277	0.00302	18.9	295	0.00032	0.00004	0.0007	12	464	0	0.00024	0.00195	28	687	0.00331	0	0.00012
17.2	144	0.00256	0.00009	0.00012	20.4	295	0.0008	0.00053	0.0007	34.2	464	0.00171	0	0	31.4	693	0.00019	0.00011	0.0003
18.3	144	0.00042	0.00073	0.0008	32.3	299	0.00028	0.00019	0.00024	23.6	467	0.00123	0.0009	0.00112	33.4	697	0.00373	0	0
15.4	146	0.00599	0.00204	0.00337	23.5	301	0.00088	0.00064	0.00098	28	472	0.00159	0.00125	0.00154	23.6	702	0.00147	0	0.00007
55.6	149	0.00907	0.00571	0.00852	19	303	0	0.00084	0.00076	19.7	474	0.00141	0	0.00006	26.7	720	0.00111	0	0.00025
12.8	152	0.00062	0	0.00016	19.5	303	0.00004	0.00083	0.00081	23.6	475	0.00096	0.0008	0.00099	23.2	726	0.00141	0	0
14.3	152	0	0.00078	0.00044	17.4	305	0.00097	0.0007	0.00104	22.3	476	0.00104	0.00082	0.00102	24.9	729	0.00039	0.00012	0
38.3	158	0.00443	0.00307	0.00435	12.3	310	0.00164	0.00276	0.01491	28	477	0.00066	0.00047	0.00068	37.4	737	0.00141	0	0
16.4	162	0.00017	0	0.00056	17.5	316	0.00038	0.00034	0.00036	21.8	480	0.0006	0	0.00004	21.9	742	0.00206	0	0
10.2	166	0.07745	0.00502	0.0218	21.2	320	0.00098	0	0	22.2	481	0.00064	0.00041	0.00069	26	749	0.00088	0.00039	0.00014
21.2	166	0.0012	0	0.00003	21.6	321	0.00171	0.0011	0.00168	34.7	492	0.00069	0.00034	0.00055	30	751	0.00467	0	0.00027
12.2	169	0	0.00086	0.00178	24.9	323	0.00101	0.00069	0.00096	26	498	0.00091	0.00062	0.00013	27.8	761	0.00412	0	0
20.7	169	0	0.00024	0.00002	18.9	327	0.00127	0.00075	0.00108	23.1	503	0.00028	0.0002	0.00024	26.3	772	0.00088	0.00056	0.00008
41.5	177	0.00066	0.00047	0.00053	21.2	328	0.00064	0	0.00004	27.3	504	0.00243	0.0002	0.00005	36.8	775	0.00424	0	0
12	178	0.00054	0.00025	0.00036	23.6	332	0.00055	0	0	24.5	506	0.00098	0.00066	0.00114	25.2	791	0.00138	0	0
21.8	182	0.00187	0	0.00002	26	332	0.00055	0.0005	0.00002	25.3	509	0.00201	0	0	38.5	796	0.00996	0	0
26.4	183	0.00254	0.00167	0.00245	20.4	335	0.0018	0.00114	0.00149	28.9	510	0.00476	0	0	21.9	803	0.00241	0	0.00002
10.1	185	0.00014	0.00089	0.00139	19.2	340	0.00192	0.00121	0.00145	24.5	511	0.00085	0.00052	0.00072	28.3	803	0.0017	0	0
19.7	185	0.00066	0.00004	0.00003	45.1	343	0.00966	0.00622	0.00865	25.7	512	0.00015	0.00015	0.00035	29.3	804	0.00259	0	0
15.4	188	0.08928	0.02719	0.04607	14.2	345	0.00579	0.00004	0	28.4	514	0.00159	0	0.00011	31.8	809	0.02907	0	0.0004
12.5	189	0.00326	0	0.00016	24.9	345	0.00111	0.00076	0.00122	26.3	515	0.00331	0.00232	0	36.9	831	0.00494	0	0
13.2	189	0.00542	0.00031	0.00075	15.2	349	0.00014	0.00375	0.008	28.8	516	0.00176	0.00107	0.00142	27.3	836	0.00278	0	0
20.4	189	0.00077	0.00034	0.00053	18.9	349	0.00109	0.00068	0.00099	19.4	518	0.00054	0	0.00005	36.9	841	0.00356	0	0
14.6	195	0	0.00042	0.00107	20.6	350	0.00212	0.00007	0.00007	23.1	520	0.0006	0.00038	0.0006	26.8	844	0.00179	0	0
55.4	195	0.00179	0.00118	0.00138	18.3	357	0.00037	0	0.00005	28.8	521	0.00062	0.00045	0.00057	27.8	853	0.01106	0	0
15.2	196	0	0.00047	0.00193	21.9	359	0.00035	0	0.00004	23.1	525	0.00037	0.00031	0.00039	37.6	855	0.00279	0	0
17.1	197	0	0.00089	0.00126	12.1	361	0.00049	0.00005	0.00005	24.5	527	0.0003	0.00018	0.00025	27.3	862	0.00189	0	0
12	199	0	0.00054	0.00066	19.2	362	0.00107	0.00074	0.00105	24.9	529	0.00475	0	0	30.8	865	0.00768	0	0
10.1	201	0	0.00062	0.00103	10.1	363	0	0.00024	0.00042	25.3	529	0.00475	0	0.0001	35.4	873	0.00966	0	0
11.2	203	0.0009	0	0	22.6	365	0.00142	0.00098	0.0014	25.3	550	0.0008	0.00057	0.00073	27.1	874	0	0.00103	0
15.3	205	0.02278	0.00639	0.01131	15.2	367	0.00013	0.00373	0.00828	30.1	554	0	0.00141	0	37.6	880	0.00259	0	0
16.3	211	0.00008	0.00023	0.00009	26.1	367	0.00111	0.00102	0.00101	29.5	560	0.00125	0.00077	0.00109	22.4	882	0.00052	0	0
37.7	212	0.00092	0.00034	0.00047	20.1	371	0.00104	0.00066	0.00091	24	564	0.00042	0.0003	0.00048	22.4	886	0.00095	0	0
12.6	215	0.00906	0.00069	0.00246	52.1	371	0.00543	0.00342	0.00471	23.4	565	0.0001	0.00166	0.00025	26	887	0.00487	0	0.00005
18.3	217	0.00033	0.0007	0.00082	19.7	375	0.00088	0	0	24.5	566	0.00038	0.00027	0.00031	24.9	893	0.00007	0	0
12	219	0.00031	0.00008	0.00035	20.2	377	0.00101	0.00082	0.00112	30.8	568	0.00651	0	0	34.6	893	0.02175	0	0
48.2	219	0.00052	0.0003	0.00042	18.5	379	0.00011	0.00039	0.0002	24	569	0.00025	0.00021	0.00039	22.4	896	0.00043	0	0
13.5	220	0.00183	0.00105	0.00224	21.6	379	0.00205	0.00123	0.00171	26.1	569	0	0.00069	0.00039	36.8	904	0.00396	0	0
9.7	222	0.00988	0.0003	0.00016	14	383	0.00147	0.00006	0.00153	22.7	570	0.01253	0.00208	0.00013	38.5	928	0.01109	0	0
10.1	225	0	0.00032	0.00037	14.6	384	0	0.00044	0.00077	22.6	571	0.00959	0.00239	0.00018	28.4	931	0.00098	0	0
39.4	225	0.00092	0.00064	0.00085	26.1	384	0.00138	0.00088	0.00119	21.2	572	0.00165	0	0.00091	28.5	961	0.00241	0	0
36.8	226	0.00081	0.00048	0.00063	20.1	388	0.00062	0.00035	0.00055	20.3	588	0.00052	0	0	33.4	985	0.00245	0	0
13.5	227	0.00075	0.00018	0.00101	26	389	0.00088	0.00061	0.00075	20.8	589	0.00006	0.00047	0.00197	27.4	988	0.00188	0	0
50	228	0.02206	0.01415	0.01947	20.1	393	0.001	0.00062	0.00092	24.5	589	0.00042	0.00026	0.00197	28.5	993	0.00292	0	0
15.6	229	0.01242	0.0012	0.00375	17.4	396	0.00027	0.00001	0.00003	27.3	593	0.00162	0.00012	0	27.8	1001	0.00458	0	0
16.4	229	0.01064	0.00036	0.00133	19.4	405	0.00031	0	0.00004	26	594	0.00062	0.00037	0.00066	30	1001	0.01423	0	0
50.7	230	0.00484	0.00302	0.00431	21.2	407	0.0023	0	0.00002	24	597	0.00739	0.00027	0.00066	25.7	1004	0.00039	0.00036	0.00001
19	233	0.00038	0.0003	0.00036	20.1	409	0.00021	0.00017	0.00099	21.8	602	0.00163	0	0	30.2	1021	0.00509	0	0.00013
45.1	240	0.00672	0.00439	0.00558	23.6	409	0.00103	0.00065	0.00099	24.1	603	0.00191	0	0	29.3	1059	0.00486	0	0
18.1	245	0.00201	0	0.00009	27.1	411	0.00068	0.00048	0.00072	30.2	605	0.00054	0.00038	0.00058	30.8	1071	0.02224	0.00161	0.00028
19.7	245	0.00079	0.00002	0.00003	24.5	413	0.00168	0	0.00002	21.8	612	0.00123	0	0	31.8	1078	0.00723	0	0
20	250	0.00089	0.00051	0.00079	21.2	415	0.00064	0.00047	0.00061	23.7	625	0.00087	0.00101	0.00019	26.8	1113	0.00221	0	0
12.5	254	0.00011	0.00177	0.00197	20.1	416	0.00019	0.00012	0.00019	28	642	0.01218	0	0	27.3	1115	0.00401	0	0
21.8	257	0.00048	0.00033	0.00063	26	416	0.0015	0.0004	0	26.1	646	0.00021	0.00026	0.00056	33.4	1149	0.00192	0	0
15.8	261	0.00105	0.00047	0.00095	12	419	0.00049	0	0.00005	30.8	648	0.00042	0.00033	0	28.3	1204	0.00105	0	0
16.3	261	0.00017	0.00016	0.00027	22.7	423	0.00148	0.00115	0.00152	26.3	651	0.0006	0.00054	0.00004	30.8	1285	0.00828	0.00052	0.00003
20.6	261	0.00162	0	0.00002	27.1	428	0.00185	0.00123	0.00143	19.4	656	0.00033	0	0					

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About the Author

Curriculum vitae

Anna Valentina Luparelli was born on January 20th, 1992, in Rome (Italy). She has got her first Bachelor's degree in Dietetics at the University "Cattolica Del Sacro Cuore" in Rome, in November 2013. She completed the rest of her academic studies at the University of Pisa getting both a second Bachelor's degree and Master's Degree respectively in Agricultural science and Food Technologies at the Department of Agricultural, Food and Agro-environmental Sciences. From March 1st, 2017, she has done a six-month training period at the University college of Dublin, Dublin, Ireland, funded by the Erasmus traineeship program. During this traineeship, she has carried on her Master's thesis project, developing several skills in the food technology field. In November 2018, she has started the PhD in Food Science at the Department of Food and Drug of the University of Parma, under the supervision of Prof. Stefano Sforza and Prof. Augusta Caligiani. Her doctoral project has been funded by Emilia Romagna region and concerned the valorization of agri-food by-products through insects. In this respect, part of her PhD has been developed in the framework of an Italian project, Bioeco-flies, financed within the Rural Development Programme of the Emilia-Romagna Region, which intended to exploit the bioconversion potential of black soldier fly *(Hermetia illucens L.)* to enhance agri-food wastes, obtaining products with a high added value. In agreement with the principle of a circular economy, also the insect wastes derived from the bioconversion process were subject of a valorization study, conducted during her second year. Her PhD research also aimed at the development of an analytical method for insect chitin characterization. From May 1st, 2021, she has worked for five months as visiting researcher at University of Rovira i Virgili (URV), Tarragona, Spain, where she studied the biological activity (with a focus on the enterhormone secretion) of Black soldier

fly through an *in vitro* interaction between *in vitro* digested insect with human intestinal tract cells, acquiring competence in the biochemical and biotechnologies sector.

She is author of scientific articles published in ranked journals.

List of publications:

- Barbi, S., Macavei, L. I., Fuso, A., **Luparelli, A. V.**, Caligiani, A., Ferrari, A. M., ... & Montorsi, M. (2020). Valorization of seasonal agri-food leftovers through insects. Science of The Total Environment, 709, 136209.

- Hadj Saadoun, J., **Luparelli, A. V.**, Caligiani, A., Macavei, L. I., Maistrello, L., Neviani, E., ... & Lazzi, C. (2020). Antimicrobial Biomasses from Lactic Acid Fermentation of Black Soldier Fly Prepupae and Related By-Products. Microorganisms, 8 (11), 1785.

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- **Luparelli, A. V.**, Leni, G., Fuso A., Palini S., Sforza S. & Caligiani A. Development of a quantitative UPLC-ESI/MS method for the simultaneous determination of the chitin and protein content in insects (submitted).

- **Luparelli, A. V.**, Hadj Saadoun, J., Lazzi C., Sforza S. & Caligiani A Fermentation by Lactobacillus spp. modifies the molecular composition of black soldier fly prepupae and-derived biomasses (submitted).

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- **Luparelli A.V.**, Hadj Saadoun J., Ricci A., Neviani E., Galaverna G., Lazzi C., Caligiani A., Sforza S. Solid state fermentation of black soldier fly puparium, prepupae and adults: tailoring the molecular composition of a residual biomass towards antimicrobial properties. RAFA Conference, 05-08/11/2019. Prague (Czech Republic)

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- **Luparelli A.V.,** Fuso A, Barbi S, Macavei L.I., Polacco A., Montorsi M., Maistrello L., Sforza S., Caligiani A. Valorization of vegetable by products by insects. La chimica degli alimenti e i giovani ricercatori, SCI. 23-24/09/2019. Milan (Italy)

- **Luparelli A.V.**, Hadj Saadoun J., Ricci A., Neviani E., Galaverna G., Lazzi C., Caligiani A., Sforza S. Search for antimicrobial molecules from lactic acid fermentation of Black Soldier Fly prepupae and related byproducts. IFW virtual conference. 23-26/11/2020. Québec

- Lolli V., Fuso A., **Luparelli A.V.,** Bonzanini F., Leni G., Sforza S., Caligiani A. The effect of the diet and killing methods on insect lipids: the case study of Black Soldier Fly (*Hermetia illucens*). 18th Euro Fed Lipid Congress and Expo, virtual conference. 18-21/10/2021.

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- General Assembly and the IPIFF Workshop. 2-3/12/2019 Brussels, Belgium

Honours and awards

- **Winner** of "EROI-S3 PhD Video Contest". ART-ER, Competenze per l'Innovazione. 30/04/2020. Parma, italy

- **Poster Prize Winner**: **Luparelli A.V.**, Fuso A, Barbi S, Macavei L.I., Polacco A., Montorsi M., Maistrello L., Sforza S., Caligiani A. Valorization of vegetable by products through insects. Valorization of by-products from agri-food supply chains for the development of functional ingredients, foods and nutraceuticals. 24-26/06/2019. San Floriano (Italy).