Tavole Rotonde sui maggiori problemi
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Sotto gli auspici del MIPAAF

XLIII.
EDIBLE INSECTS: FROM BIOLOGY TO APPLICATIONS

Estratto da:
ATTI DELLA
ACCADEMIA NAZIONALE
ITALIANA DI ENTOMOLOGIA
Anno LXX - 2022

CEREBRO FAVCIBVS VTERO
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Tavola Rotonda su:

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Tavola Rotonda su:

EDIBLE INSECTS: FROM BIOLOGY TO APPLICATIONS

Coordinatori:

Morena Casartelli Accademica

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PROGRESS AND CHALLENGES OF INSECTS AS FOOD AND FEED

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Progress and challenges of insects as food and feed

An overview is given on recent developments concerning insects as food and food. Most production is targeted towards pet food, but in the next two decades it will shift to aquafeed. More than 80% of all publications dealing with edible insects have appeared during the last six years. Genetics are increasingly utilized to improve production. The environmental impact of producing insects compares well to other alternative proteins, in particular their capacity to degrade organic waste streams. Edible insects are not only a good source of nutrients but also seem to provide health benefits, not only for humans and animals but also for plants (the left-over substrate). The challenge of convincing Western consumers is reviewed and whether sustainability is an issue. Processing techniques are being developed. The sector of insects as food and feed is developing fast, thanks also to an increasingly conducive legislative framework.

KEY WORDS: edible insects, biodegradation, black soldier fly, organic waste streams, health

SYNTHESIS OF LECTURE

The recent interest in insects as food and feed started after the FAO publication "Edible insects: future prospects of insects as food and feed" (VAN HUIS *et al.*, 2013). The sudden interest in alternative proteins was very likely triggered by environmental concerns about meat production. The academic interest is increasing: More than 85% of all publications on edible insects have been published during the last six years. Concerning the market: in 2021, 54% of insect products went to pets and 17% to aquafeed; in 2030 this will be 30 and 40%, respectively; for poultry it remains at 25% and for pigs 4% (DE JONG and NIKOLIK, 2021). One problem is the high price, but it is expected that it will go down in this period by about 50%.

We need alternative protein sources because the land area for livestock (now 80%) cannot satisfy the increasing demand. Moreover, livestock contributes to 15% of all greenhouse gas (GHG) emissions, beef having the highest impact in terms of GHG emissions and land use. The production of insects produces less GHG and requires less land and water than the production of meat (MIGLIETTA *et al.*, 2015; OONINCX and DE BOER, 2012). Another big advantage of insects is that they can bio-transform organic waste into high value protein. Also, it seems that polystyrene can be degraded by the yellow mealworm and black soldier fly (BSF) larvae and their gut microbes. Any kind of organic waste can be handled by the BSF larvae, even manure, although this is not yet allowed in the EU.

Up till now no diseases have been detected for the BSF (JOOSTEN *et al.*, 2020). For crickets however, many viruses are present, although it is possible to shift to other cricket species not susceptible to a particular disease. There is now an EU project that is training approx. 15 PhD students in veterinary science for insects.

The nutritional value of insect products is comparable to meat products, and there may be health benefits for humans (VAN HUIS *et al.*, 2021). There are also health benefits for animals. For example, from BSF larvae 68 anti-microbial compounds (AMPs) have been found, of which 57 have antimicrobial, antiviral, and or antifungal activity (MORETTA *et al.*, 2020). No drug resistance has been found among bacteria in livestock, and it may therefore be an interesting alternative to antibiotics in animal diets (XIA *et al.*, 2021).

Depending on the animal species, fishmeal or soy can be partially replaced by insects (VAN HUIS and GASCO, 2023). Insect frass not only has an effect as fertilizer, but it also increases crop resistance through the beneficial effect of insects' exuviae and frass on soil microbes (BARRÁGAN-FONSECA *et al.*, 2022).

The price of insects as animal feed is still too high; it would be better to eat mealworms directly instead of eating chickens fed with mealworms. However, the price will come down, depending mainly on whether cheap and reliable quantities of unused side-streams are available in large quantities.

Concerning consumer attitudes, the main challenge is persuading consumers to go from the occasional snack to including insects as a regular part of their diet. About 20-

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25% of Western consumers are willing to try insect-based products. As disgust is the main problem, it has been suggested to target people who have low levels of disgust and high levels of sensation-seeking. Other strategies to convince consumers are deliciousness, providing information, giving people a taste experience, and disguising the product in familiar products such as bread, pastas, burgers, sausages, etc. The sustainability argument does not seem to convince the consumer.

Processing can be done by dehydrating, roasting and grinding the insect into powder/flour. A thermal treatment is necessary for decontamination. The powdered food can be added to crackers, pasta, energy bars (5-20% crickets), snacks, burgers, meatballs (Reverberi, 2021). A problem to be solved is the lipid content, which influences flavor and odor. Also the proteins, fat and chitin can be isolated using several pathways (El Hajj *et al.*, 2022). There are several industrial applications apart from using the insects as food and feed: biodiesel or fuel from BSF lipids, cosmetic and skincare products from purified BSF fat to improve skin conditions such as smoothing, revitalizing, moisturizing, and tightening (Van Huis, 2022b).

Concerning food safety, bioaccumulation may occur, such as cadmium in BSF larvae and arsine in the yellow mealworm, a feature which can also be used in bioremediation procedures. Mycotoxins and polycyclic aromatic hydrocarbons do not seem to accumulate, and mycotoxins and some veterinary drugs can be degraded by insects (Alagappan *et al.*, 2022) with the help of symbiotic microbes. Concerning biological contaminants, some bacteria may be dangerous, while we do not know much about fungi, viruses, protozoa and prions. Processing can reduce pathogens. Legislation is becoming increasingly conducive in the EU: insects were allowed for aquafeed in 2017, and for poultry and pigs in 2021. Manure (although it can reduce ammonia pollution) and catering waste are not yet allowed to be used as substrate for insects to be used as feed. Since 2022, several insect products have been allowed as food, *i.e*. those containing house cricket, yellow mealworm and migratory locust.

Insect welfare needs to be taken seriously as more evidence is starting to emerge about insects as ‘sentient beings’. The sector of insects as food and feed is progressing rapidly and depends also on the (inter)national collaboration between research, private enterprise and the government. This promotes innovation, attracts talent and devises solutions for the challenges of tomorrow (Van Huis, 2022a).

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STUDIO DELLA FISIOLOGIA DELL'INTESTINO MEDIO DI *HERMETIA ILLUCENS*

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Insights into the physiology of Hermetia illucens *midgut*

The larvae of *Hermetia illucens* (*Diptera*, *Stratiomyidae*) are promising agents of bioconversion and valorisation of organic waste and by-products of the agri-food production chain. From the insect biomass significant amount of proteins, lipids, and bioactive molecules, such as antimicrobial peptides and chitin, can be obtained and used as row materials or additives for the production of feed for monogastric animals, bioplastics, biodiesel, cosmetics or medical products. Therefore, this insect is suitable to build circular economy supply chains and make waste management and production processes increasingly sustainable. Solid knowledge about the physiology of the larvae and, in particular, about the morphofunctional features of the midgut, which is the organ directly involved in digestion and absorption of nutrients, is essential to improve their bioconversion capabilities. For this reason, we performed an in depth characterization of the larval midgut. Our studies showed that the midgut is an extremely complex organ that has a strong morphofunctional regionalization and is able to set in motion post-ingestion regulatory mechanisms to exploit nutritionally poor rearing substrates. This functional plasticity contributes to the capability of these larvae to grow on a variety of feeding substrates even when their nutritional content is low. We also characterized the midgut microbiota. We demonstrated that the diet as well as the physico-chemical features of the three regions in which the midgut can be divided stongly shape the residing microbiota and that the bacterial community present in the posterior tract of the midgut could play a relevant role in the host physiology. With the aim of having a complete picture of the digestive system of *H. illucens*, we also analyzed its characteristics in the adult insect. Although the literature frequently reportes that *H. illucens* flies are not able to eat, our data unequivocally demonstrated that they possess a functional digestive system and that food administration affects their longevity. These data open up the possibility to manipulate the feeding substrate of the fly to improve its performances in mass rearing procedures.

Key Words: Black soldiers fly, bioconversion, midgut physiology, microbiota

Le larve di *Hermetia illucens* (Diptera, Stratiomyidae) (Fig. 1) sono considerate promettenti agenti di bioconversione e valorizzazione di rifiuti organici e sottoprodotti della filiera agroalimentare perché sono saprofaghe, hanno un elevato tasso di consumo del substrato di crescita e un rapido sviluppo. Inoltre, dalle larve mature è possibile estrarre significative quantità di proteine, lipidi e molecole bioattive, come peptidi antimicrobici o chitina, che possono essere impiegate come materie prime o additivi (a seconda del tipo di substrato utilizzato per allevare l'insetto, nel rispetto delle normative vigenti) per la produzione di mangimi per animali monogastrici, bioplastica, biodiesel, cosmetici o prodotti medicali (Surendra *et al.,* 2020; Siddiqui *et al.*, 2022). Inoltre, il residuo dell'allevamento può essere valorizzato come fertilizzante organico o per la produzione di biogas (Surendra *et al.,* 2020). Questo insetto offre quindi l'opportunità di creare filiere di economia circolare per rendere la gestione di rifiuti e sottoprodotti, così come i processi produttivi sempre più sostenibili, in linea con la strategia "Farm to Fork", uno dei pilastri del Green Deal europeo (Cappellozza *et al.*, 2019).

Circa sette anni fa, quando il nostro gruppo di ricerca ha iniziato a studiare questo insetto in collaborazione con quello del prof. Gianluca Tettamanti dell'Università dell'Insubria, erano note poche informazioni sulla sua fisiologia. In letteratura erano riportate informazioni essenzialmente sui metodi per allevare questo dittero, sull'efficienza delle larve di biotrasformare differenti tipologie di scarti e rifiuti, sulla composizione chimica delle larve in funzione della tipologia di substrato di crescita e sulle pos-

Fig. 1 - Larve di *Hermetia illucens*

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sibili applicazioni delle macromolecole estratte dall'insetto. Abbiamo quindi focalizzato l'attenzione sull'apparato digerente e, in particolare, sull'intestino medio, ossia la regione deputata alla digestione e all'assorbimento dei nutrienti e, pertanto, direttamente responsabile del processo di bioconversione. Infatti, solo attraverso un'approfondita conoscenza di questo organo è possibile comprendere le basi fisiologiche della straordinaria plasticità alimentare di queste larve e sfruttarne al meglio la capacità di bioconversione.

In primo luogo abbiamo eseguito una fine caratterizzazione morfofunzionale dell'intestino medio delle larve di *H. illucens* (Bonelli *et al.,* 2019) allevate su dieta standard, una dieta ottimale per il loro sviluppo, contenente crusca di frumento, farina di mais ed erba medica (Hogsette, 1992). Da questo studio è emerso che l'intestino medio è estremamente complesso e può essere suddiviso in tre tratti – anteriore, intermedio e posteriore – ognuno dei quali presenta specifiche caratteristiche morfologiche e funzionali. Il tratto anteriore, che ha un pH luminale debolmente acido, è costituito da cellule colonnari con caratteristiche ultrastrutturali tipiche delle cellule secretorie (reticolo endoplasmatico rugoso molto sviluppato, numerosi mitocontri e vescicole nella porzione apicale della cellula). In questo tratto sono attive amilasi e lipasi in grado di digerire, rispettivamente, polisaccaridi e lipidi. Il tratto intermedio può essere suddiviso in due porzioni: la prima, piuttosto breve, presenta un epitelio in cui sono presenti le cellule cuprofiliche, nella membrana apicale di queste cellule si trova una proteina che media il trasporto attivo di ioni H^+ nel lume (H^+ V-ATPasi) responsabile del pH fortemente acido presente nel lume tratto intermedio dell'intestino medio; nella seconda porzione l'epitelio è formato da cellule piuttosto appiattite, il lume intestinale è più ampio rispetto a quello degli altri tratti e l'unica attività enzimatica presente è quella del lisozima. Le caratteristiche dell'intestino intermedio appena descritte, in particolare il pH luminale estremamente acido (intorno a 2 unità di pH) e la presenza del lisozima, suggeriscono che questo tratto abbia l'importante ruolo di uccidere i microrganismi patogeni ingeriti con la dieta. Anche il tratto posteriore dell'intestino medio, che è il più esteso dei tre e presenta un lume alcalino, può essere suddiviso in due pozioni: la prima presenta cellule colonnari con caratteristiche simili a quelle del tratto anteriore; nella seconda le cellule colonnari hanno microvilli più sviluppati di quelli presenti negli altri tratti, caratteristica che indica un importante ruolo del tratto posteriore dell'intestino medio nell'assorbimento dei nutrienti. Inoltre, qui avviene la digestione delle proteine grazie all'attività di endopeptidasi, in particolare serin-proteasi come tripsina e chimotripsina, ed esopeptidasi, come amminopeptidasi, ancorate alla membrana apicale delle cellule colonnari, e prosegue la digestione di polisaccaridi e lipidi grazie ad amilasi e lipasi. Il tratto posteriore è quindi la principale sede della digestione e dell'assorbimento dei nutrienti. Dalla caratterizzazione dell'intestino medio delle larve di *H. illucens* è emerso che questo organo è estremamente complesso e presenta una forte "regionalizzazione" morfofunzionale (Fig. 2).

Questo primo studio ha rappresentato il punto di partenza per indagare se le larve di *H. illucens* siano in grado di sfruttare substrati di crescita poveri dal punto di vista nutrizionale grazie a meccanismi di regolazione post-ingestione messi in atto dall'intestino medio. Infatti, l'efficacia di questi meccanismi, unita alla capacità di regolare l'ingestione di cibo, sono fondamentali per poter compensare la variabilità nella composizione della dieta e soddisfare le esigenze nutrizionali dell'insetto. Le larve sono state quindi allevate con dieta standard, utilizzata come controllo, e con una dieta contenente solo frutta e verdura, un substrato di crescita che mima uno scarto della filiera agroalimentare e con un ridotto contenuto di proteine, lipidi e carboidrati rispetto alla dieta standard (Bonelli *et al.*, 2020). Sebbene le larve abbiano performance di crescita migliori su quest'ultima in termini di durata dello stadio larvale e massimo peso raggiunto prima dell'impupamento, non si sono osservate differenze nella sopravvivenza delle larve e nel numero di adulti sfarfallati con le due diete. Lo studio da noi eseguito ha messo in evidenza che le larve, attraverso la modulazione dell'attività dell'intestino medio, sono in grado di sfruttare efficaciemente substrati di crescita poveri dal punto di vista nutrizionale (Bonelli *et al.*, 2020). In particolare, abbiamo osservato che l'attività digestiva viene finemente regolata: l'attività delle proteasi (endopeptidasi e esepeptidasi) aumenta in modo significativo quando le larve sono allevate con dieta contenente solo frutta e verdura rispetto a quanto osservato con dieta standard. Questo dato suggerisce che l'insetto riesce a ottenere un adeguato apporto di amminoacidi anche se allevato con una dieta povera di proteine grazie alla modulazione degli enzimi coinvolti nella loro digestione. L'analisi trascrittomica ha confermato che molti geni che codificano per enzimi coinvolti nella digestione delle proteine sono sovraespressi nelle larve alimentante con la dieta contenente solo frutta e verdura. Inoltre, queste larve mostrano adattamenti morfologici dell'epitelio del tratto posteriore dell'intestino medio, quello maggiormente coinvolto nei processi di assorbimento dei nutrienti. In particolare i microvilli delle cellule colonnari risultano più allungati rispetto a quanto osservato nel medesino tratto delle larve allevate su dieta standard. Questa evidenza indica una maggiore superficie a disposizione per i processi di assorbimento. Possiamo concludere che l'intestino medio delle larve di *H. illucens* è un organo funzionalmente plastico che sicuramente contribuisce alla capacità di questo insetto di sfruttare efficaciemente substrati di crescita con contenuti nutrizionali diversi. Intendiamo proseguire la ricerca caratterizzando i meccanismi biochimici e molecolari alla base della plasticità dell'intestino medio. Questi meccanismi possono coinvolgere altri tessuti, come il corpo grasso e il sistema nervoso, ma anche il microbiota intestinale che potrebbe svolgere un ruolo importante nei processi digestivi e nel supportare la larva dal punto di vista nutrizionale.

Nei nostri studi sulla caratterizzazione funzionale dell'intestino medio delle larve di *H. illucens* abbiamo considerato anche il microbiota intestinale (Bruno *et al.,* 2019b). In particolare, abbiamo analizzato l'effetto di tre diverse diete (dieta standard, dieta contenente solo frutta e verdura, dieta con elevato contenuto proteico contenente farina di pesce) sulla composizione del microbiota presente nei tre tratti in cui è possibile suddividere l'intestino medio (anteriore, intermedio e posteriore). Infatti, non solo la composizione della dieta, ma anche le diverse caratteristiche chimico-fisiche presenti in queste tre regioni potrebbero avere un forte impatto sulla composizione della comunità batterica intestinale. I nostri risultati hanno effettivamente messo in evidenza che il microbiota è influenzato dalle caratteristiche dei tre tratti dell'intestino medio. In particolare, per tutte le diete la carica batterica è simile nelle regioni anteriore e intermedia, mentre aumenta nel tratto posteriore dell'intestino medio. Inoltre, per tutte le diete il tratto anteriore è caratterizzato dalla più alta diversità batterica la quale progressivamente diminuisce andando verso il tratto posteriore, dove probabilmente ci sono le condizioni ottimali per la crescita di una comunità batterica composta da un numero relativamente limitato di specie che possono essere considerate i veri simbionti di questo insetto. È possibile che il pH fortemente acido e l'attività del lisozima presenti nel lume del tratto intermedio svolgano un ruolo importante in questo processo di selezione. Per quanto riguarda la composizione del microbiota, i *phyla* con il maggior numero di specie nei tre tratti dell'intestino medio delle larve allevate con dieta standard e con dieta contenente solo frutta e verdura sono gli stessi e hanno abbondanze relative simili (il *phylum* dominante è *Bacteroidetes*), mentre i *phyla* prevalenti nelle larve allevate con dieta contenente farina di pesce sono *Proteobacteria* e *Firmicutes*. Anche scendendo a un rango tassonomico inferiore, il genere, nel tratto posteriore dell'intestino medio le larve allevate con dieta standard o con dieta contenente frutta e verdura hanno un microbiota simile, mentre nelle larve allevate con dieta contenente farina di pesce il microbiota presenta una composizione completamente diversa, in cui proteobatteri del genere *Providencia* rappresentano il *taxon* con la maggiore abbondanza relativa. Poiché molti patogeni opportunisti appartengono a questo genere, non si può escludere che diete conteneti elevate quantità di proteina, come quella utilizzata in questo studio in cui le proteine rappresentano più del 60% del peso secco, possano causare disbiosi nelle larve, ipotesi supportata anche dalla ridotta performance di crescita dell'insetto

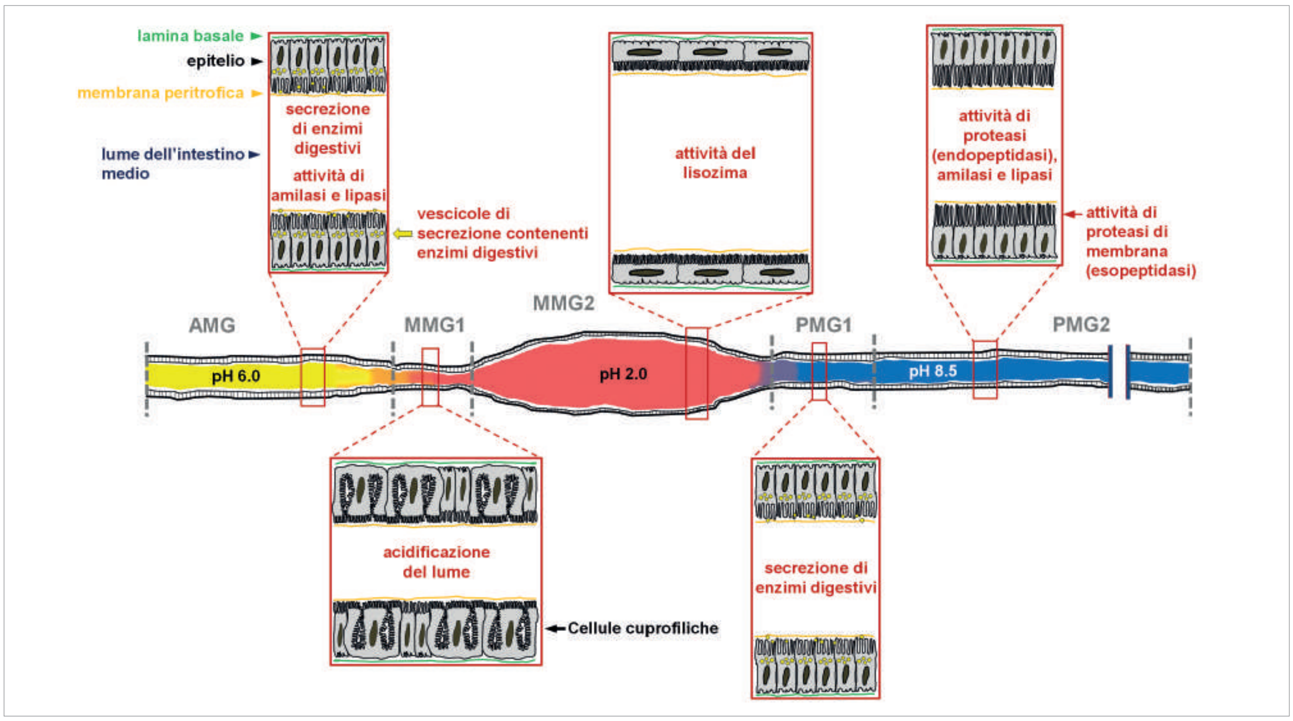

Fig. 2 - Rappresentazione schematica dell'intestino medio delle larve di *H. illucens* in cui sono riportate le principali caratteristiche morfofunzionali dei tre tratti in cui questo organo può essere suddiviso. Il tratto anteriore dell'intestino medio (AMG), che ha un pH luminale acido, è caratterizzato da cellule colonnari con attività secretoria; in questa regione inizia la digestione di polisaccaridi e lipidi grazie all'attività, rispettivamente, di amilasi e lipasi. Le cellule cuprofiliche, localizzate nella prima porzione del tratto intermedio (MMG1), sono responsabili del pH fortemente acido del lume intestinale di questa regione; l'epitelio della seconda porzione del tratto intermedio (MMG2) è formato da cellule appiattite, qui non avvengono processi digestivi, ma l'attività del lisozima e il pH acido (pH = 2) del lume potrebbero rivestire un ruolo importante nell'uccidere i microrganismi patogeni ingeriti con la dieta. Il tratto posteriore dell'intestino medio è coinvolto nella digestione delle proteine grazie alla presenza di endopeptidasi e esopeptidasi, ed è responsabile dell'ulteriore digestione di polisaccaridi e lipidi. La prima porzione di questo tratto (PMG1) è caratterizzata da cellule colonnari con attività secretoria, mentre la seconda (PMG2) presenta cellule colonnari con microvilli più lunghi rispetto a quelli degli altri tratti, evidenza che suggerisce un importante ruolo di queste cellule nell'assorbimento dei nutrienti. Modificato da Bonelli *et al.*, 2019.

su questa dieta. Questo è un aspetto importante che deve essere considerato nella formulazione di diete per l'allevamento massale di questo insetto. Questo studio, che è stato uno dei primi pubblicati sul microbiota intestinale delle larve di *H. illucens*, ha messo in evidenza che le proprietà chimico-fisiche dell'intestino medio condizionano la composizione della comunità batterica e che i batteri che risiedono nel tratto posteriore potrebbero avere un ruolo rilevante nella fisiologia dell'ospite, aspetto che merita ulteriori indagini.

Con l'obiettivo di avere un quadro completo sull'apparato digerente di *H. illucens*, abbiamo analizzato le sue caratteristiche anche nell'insetto adulto (Bruno *et al.*, 2019a). Senza il supporto di studi morfologici e funzionali, in letteratuta è frequentemente riportato che le mosche di questa specie (Fig. 3) non si nutrono, non possono essere quindi vettori di agenti patogeni, e che le loro performance dipendono esclusivamente dalle riserve accumulate durante lo stadio larvale (a titolo di esempio: Gobbi *et al.*, 2013; Sheppard *et al.*, 1994; Sheppard *et al.*, 2002; Tomberlin *et al.*, 2009; Tomberlin e Sheppard 2002; Tomberlin *et al.*, 2002). Solo un numero limitato di studi dimostra che la longevità e le performance riproduttive degli adulti dipendono dal tipo di substrato messo a loro disposizione (Bertinetti *et al.*, 2019; Lupi *et al.*, 2019; Nakamura *et al.*, 2016), senza però esaminare le proprietà funzionali dell'apparato digerente di

Fig. 3 - Adulto di *Hermetia illucens*

questo stadio.

Le nostre ricerche (Bruno *et al.*, 2019a) hanno dimostrato, utilizzando differenti approcci, che l'apparato digerente viene completamente rimodellato durante la metamorfosi e che l'insetto adulto è in grado di ingerire il cibo, il cibo ingerito transita lungo l'intestino e vengono eliminate le frass. Inoltre, nell'intestino medio dell'adulto sono attivi enzimi in grado di digerire proteine e zuccheri. Infine, abbiamo osservato che le mosche possono effettivamente sopravvivere senza nutrirsi, ma è possibile migliorare la durata dello stadio adulto e le performance riproduttive se l'insetto viene alimentato con sostanze zuccherine. Questi dati non solo dimostrano che l'adulto di *H. illucens* è in grado di nutrirsi perché dotato di un canale alimentare perfettamente funzionale, ma mettono in evidenza che è possibile migliorarne le performance riproduttive scegliendo adeguati substrati per la sua alimentazione, aspetto sicuramente rilevante per l'allevamento massale di questo insetto. Una recente review mette proprio in evidenza l'importanza di studiare lo stadio adulto di *H. illucens* per sfruttare al meglio le potenzialità di questo insetto (Lemke *et al.*, 2023).

Concludendo, è bene sottolineare l'importanza di proseguire gli studi sulla fisiologia dell'apparato digerente di *H. illucens* (Tettamanti *et al.*, 2022), ma più in generale sulla sua biologia, per rendere i processi di bioconversione mediati da questo insetto non solo efficienti ma anche sicuri. In particolare, considerando la natura dei substrati che possono essere utilizzati per l'allevamento delle larve è indispensabile valutare aspetti come la possibile presenza di contaminanti di origine biotica e abiotica nella dieta, la risposta dell'insetto a tali contaminanti, la sua capacità di accumulare molecole potenzialmente tossiche o veicolare agenti patogeni.

Riassunto

Le larve di *Hermetia illucens* (*Diptera*, *Stratiomyidae*) sono considerate promettenti agenti di bioconversione e valorizzazione di rifiuti organici e sottoprodotti della filiera agroalimentare. Dalla biomassa ottenuta è possibile estrarre significative quantità di proteine, lipidi e molecole bioattive che trovano impiego in diversi ambiti, come la produzione di mangimi per animali monogastrici, bioplastica, biodiesel, cosmetici o prodotti medicali. Questo insetto offre quindi l'opportunità di creare filiere di economia circolare per rendere la gestione di rifiuti e sottoprodotti, e i processi produttivi sempre più sostenibili. Per sfruttare al meglio la capacità di bioconversione di questo insetto è fondamentale disporre di solide conoscenze sulla sua fisiologia e, in particolare, sulle caratteristiche morfofunzionali dell'intestino medio, ossia l'organo direttamante coinvolto nella digestione e nell'assorbimento dei nutrienti. Dai nostri studi è emerso che l'intestino medio delle larve di *H. illucens* è un organo estremamente complesso che presenta una forte "regionalizzazione" morfofunzionale ed è in grado di mettere in atto meccanismi di regolazione post-ingestione che sono fondamentali per poter compensare la variabilità nella composizione della dieta. Questa plasticità funzionale garantisce di soddisfare le esigenze nutrizionali dell'insetto anche quanto il substrato di crescita ha un ridotto contenuto nutrizionale. Nei nostri studi sulla caratterizzazione funzionale dell'intestino medio delle larve di *H. illucens* non poteva mancare il microbiota. Abbiamo dimostrato che la dieta così come le caratteristiche chimico-fisiche presenti nei differenti tratti dell'intestino medio condizionano la composizione del microbiota intestinale e che i batteri che risiedono nel tratto posteriore dell'intestino medio potrebbero avere un ruolo rilevante nella fisiologia dell'ospite. Con l'obiettivo di avere un quadro completo dell'apparato digerente di *H. illucens*, abbiamo analizzato le sue caratteristiche anche nell'insetto adulto. Sebbene in letteratura venga frequentemente riportato che la mosca non si alimenta, i nostri dati indicano non solo che il suo apparato digerente è perfettamente funzionale, ma mettono in evidenza

che è possibile migliorarne le performance riproduttive scegliendo adeguati substrati per la sua alimentazione, aspetto sicuramente rilevante per l'allevamento massale di questo insetto.

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IL SISTEMA IMMUNITARIO DELLA MOSCA SOLDATO, *HERMETIA ILLUCENS*

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Defense against bacterial pathogens: spotlight on the immune system of black soldier fly

The larvae of *Hermetia illucens*, also known as black soldier fly (BSF), are gaining increasing interest worldwide for the biotransformation of organic waste. Since they live on decaying substrates, potentially rich in pathogens that can challenge their health status, growth, and reproductive performance, these larvae have presumably evolved a sophisticated and efficient immune system. This is a relevant topic, still poorly known, that can impact on the mass rearing of the larvae in production plants and can also affect the microbiological quality of the insect meal. Here we summarize the recent knowledge on the immune response of BSF larvae, focusing the attention on the two branches of the immune system, i.e., the cellular and the humoral response.

Key Words: Hemocytes, *Hermetia illucens*, Humoral response, Insect-mediated bioconversion, Waste management

Le larve del dittero *Hermetia illucens*, comunemente noto come mosca soldato, vengono allevate su svariati rifiuti organici. Tali substrati, spesso in decomposizione, possono portare gli insetti a contatto con potenziali agenti patogeni, compromettendone lo stato di salute, la crescita e le prestazioni riproduttive, con conseguenti effetti negativi sulla qualità microbiologica della farina che viene ottenuta dalle larve. D'altra parte, il particolare stile di vita e il comportamento alimentare di queste larve hanno presumibilmente contribuito a plasmare l'evoluzione di un sistema immunitario sofisticato ed efficiente in questo insetto. Questi aspetti sono estremamente rilevanti nell'ottica di produrre farine di insetto microbiologicamente sicure per il settore della mangimistica e possono avere significative ricadute applicative per quanto concerne l'allevamento massale delle larve di *H. illucens* negli impianti produttivi (Surendra, *et al.*, 2020).

Sebbene negli ultimi anni il numero di articoli relativi a *H. illucens* sia cresciuto esponenzialmente (Tettamanti *et al.*, 2022), solo pochi hanno indagato i meccanismi utilizzati dalle larve per far fronte ai patogeni, focalizzando l'attenzione sui composti antimicrobici prodotti dalle larve (Elhag *et al.*, 2017; Moretta *et al.*, 2020; Park *et al.*, 2015; Vogel *et al.*, 2018) o esplorando i meccanismi che sottendono la risposta immunitaria (von Bredow *et al.*, 2022; Zdybicka-Barabas *et al.*, 2017). Pertanto, questo aspetto rimane ancora largamente sconosciuto.

La risposta immunitaria degli insetti è basata su sofisticati meccanismi cellulari e umorali che vengono attivati dall'ingresso di patogeni nell'emocele in tempi rapidi e operano in sinergia per inattivare e rimuovere efficacemente gli agenti estranei (non self) dal corpo dell'animale. I principali protagonisti della risposta immunitaria sistemica sono gli emociti, cellule circolanti coinvolte nella fagocitosi, incapsulamento e nodulazione di corpi estranei. Queste cellule sono supportate nella loro attività dal corpo grasso, un organo che svolge svariate funzioni, tra cui la produzione di peptidi antimicrobici e altre molecole solubili (Eleftherianos *et al.*, 2021a; 2021b).

Il nostro gruppo di ricerca ha recentemente intrapreso una caratterizzazione delle componenti cellulare e umorale del sistema immunitario delle larve di *H. illucens*, esaminando inizialmente il ruolo e le modificazioni morfofunzionali degli emociti in risposta a infezione e, in secondo luogo, analizzando l'attività di alcuni componenti chiave della risposta umorale. A tale scopo, le larve sono state infettate con *Micrococcus luteus* ed *Escherichia coli*, due microrganismi che sono presenti nei substrati organici di scarto utilizzati per l'allevamento di queste larve, e rappresentano ottimi modelli per studiare la risposta immunitaria dell'insetto contro batteri Gram-positivi e Gram-negativi, o iniettate con bead cromatografiche di diverso tipo per esaminare il processo di incapsulamento. I risultati qui riportati in sintesi sono descritti nel dettaglio in due recenti pubblicazioni (Bruno *et al.*, 2021; 2022).

Il confronto iniziale dell'attività antimicrobica dell'emolinfa tal quale, contenente gli emociti e la componente umorale, con quella dell'emolinfa privata degli emociti, in larve infettate con il mix batterico, ha dimostrato che: 1) è necessaria l'azione sinergica delle due componenti del sistema immunitario per ottenere una rimozione rapida e completa dei batteri, in quanto i fattori umorali da soli non sono in grado di eliminare completamente i batteri dal corpo dell'insetto anche in tempi lunghi; 2) *M. luteus*, e probabilmente i batteri Gram-positivi in genera-

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le, è caratterizzato da una maggiore persistenza nel corpo dell'insetto rispetto a *E. coli*, come dimostrato dell'attività antimicrobica prolungata nel corso del tempo in presenza di questo batterio.

Queste evidenze preliminari hanno rappresentato la base di partenza per un'analisi dettagliata delle due componenti del sistema immunitario.

Per quanto riguarda la risposta cellulare, sono stati identificati sei tipi di emociti che collaborano nella risposta immunitaria di queste larve (Fig. 1, A-F). I proemociti rappresentano i precursori degli emociti differenziati, mentre plasmatociti, lamellociti, crystal cell, granulociti e adipoemociti costituiscono le cellule effettrici. I plasmatociti intervengono in risposta ai batteri attivando processi di fagocitosi in tempi rapidi dall'infezione, come dimostrato da esperimenti con batteri Gram-positivi e Gram-negativi fluorescenti che vengono fagocitati già cinque minuti dopo la loro iniezione nell'emocele (Fig. 1, G). Analisi di microscopia elettronica hanno confermato questa evidenza, consentendo di visualizzare le diverse fasi del processo di fagocitosi. L'attività fagocitaria aumenta progressivamente fino a un'ora, quando si osserva la completa rimozione dei batteri dall'emocele. Questa evidenza avvalora i risultati relativi all'attività antimicrobica dell'emolinfa, sopra descritti, circa l'importanza della risposta cellulo-mediata per dare luogo a una difesa immunitaria rapida ed efficace. Inoltre, in accordo con quanto osservato per l'attività antimicrobica, i batteri Gram-positivi richiedono un lasso di tempo maggiore rispetto ai Gram-negativi per essere eliminati dall'insetto. Nel corso della risposta cellulo-mediata, si assiste a un turnover degli emociti. In particolare, le cellule già circolanti nell'emocele rispondono immediatamente all'infezione batterica attivando la fagocitosi. Completato il processo di fagocitosi, queste cellule vengono eliminate tramite apoptosi, come dimostrato dall'analisi morfologica degli emociti e dalle analisi al citofluorimetro che evidenziano un incremento del tasso apoptotico un'ora dopo l'inizio dell'infezione. A partire dalle due ore, inizia la produzione *ex novo* di emociti da parte degli organi ematopoietici, portando a un incremento del numero di cellule circolanti per meglio fronteggiare l'infezione, come dimostrato dagli esperimenti di proliferazione con marcatori mitotici (istone 3 fosforilato) (Fig. 1, H).

Gli emociti sono responsabili anche della rimozione di corpi estranei di grosse dimensioni che penetrano nell'emocele, tramite il processo di incapsulamento. Esperimenti con bead cromatografiche mostrano come l'incapsulamento coinvolga granulociti, plasmatociti e lamellociti. Queste cellule proliferano e i granulociti rilasciano i propri granuli citoplasmatici, contribuendo a richiamare le altre cellule verso il corpo estraneo. In tal modo, le varie tipologie di emociti formano una capsula multistratificata e compatta che circonda il corpo estraneo, isolandolo dal punto di vista trofico e portandolo a morte (Fig. 1, I, J). Il processo di incapsulamento prevede il supporto delle crystal cell, che proliferano e rilasciano le inclusioni cristalline citoplasmatiche per produrre melanina a livello della capsula. Un aspetto importante è che la cinetica di incapsulamento e la deposizione di melanina per l'isolamento dell'agente estraneo sono influenzate significativamente dalla matrice e dalla carica della bead. Ad esempio, bead di agarosio, neutre, vengono solo parzialmente circondate dalle cellule immunitarie senza causare la produzione di melanina, mentre bead di Sephadex, cariche positivamente, sono completamente incapsulate dagli emociti e melanizzate.

Per quanto riguarda la risposta umorale sono state condotte analisi sul lisozima, un enzima in grado di idrolizzare il peptidoglicano, il componente principale della parete cellulare dei batteri Gram-positivi (Eleftherianos *et al.*, 2021a). L'attività di questo enzima aumenta progressivamente nel tempo a partire dal momento dell'infezione. Tale attività enzimatica è supportata dalla trascrizione negli emociti del gene che codifica per questo fattore, a tempi brevi dall'infezione. Va sottolineato che questo componente dell'attività umorale interviene più tardivamente rispetto all'attività cellulare, ma si protrae per tempi prolungati. Questa cinetica può essere spiegata con la necessità di rimuovere efficacemente *M. luteus* che, come riportato in precedenza, è più resistente all'attacco del sistema immunitario di *H. illucens*; in alternativa, l'attività del lisozima protratta nel tempo potrebbe consentire di prolungare la risposta immunitaria o di rispondere più rapidamente a una seconda infezione.

Una tempistica simile a quella del lisozima è stata osservata per i peptidi antimicrobici (AMPs), piccoli peptidi in grado di alterare l'integrità dei batteri portandoli a morte, contribuendo in tal modo all'attività antimicrobica del lisozima (Zhang *et al.*, 2021). Nel dettaglio, sono stati analizzati negli emociti e nel corpo grasso i livelli di espressione di Diptericina e Defensina, due AMPs che sono attivi, rispettivamente, contro i batteri Gram-negativi e Gram-positivi. Gli emociti sintetizzano entrambi gli AMPs subito dopo l'infezione e questa prima fase di produzione di AMPs è supportata da una successiva, ma prolungata, produzione di AMPs nel corpo grasso. L'espressione prolungata della Defensina, che ha un'azione più marcata sui batteri Gram-positivi, avvalora ulteriormente l'ipotesi di una maggiore resistenza di questi batteri nelle larve di *H. illucens*.

L'ultimo marcatore analizzato è il sistema della profenolossidasi (proPO), una cascata enzimatica la cui attivazione culmina nella produzione di melanina (Eleftherianos *et al.*, 2021a). Inaspettatamente la dose di batteri utilizzata negli esperimenti precedenti determina l'inibizione di questo sistema enzimatico mentre, in presenza di una carica batterica più elevata, il sistema proPO viene attivato. Pertanto, sembra che, in presenza di una bassa concentrazione di batteri nell'emolinfa, il sistema proPO sia inibito attivamente e, in tal caso, altri mediatori del sistema immunitario quali emociti, lisozima e AMPs, vengano utilizzati per rispondere all'infezione. Oltre una determinata concentrazione soglia di batteri, il sistema proPO viene attivato, portando alla produzione e al rilascio di melanina nel corpo della larva per contrastare la massiccia infezione e supportare la risposta immunitaria.

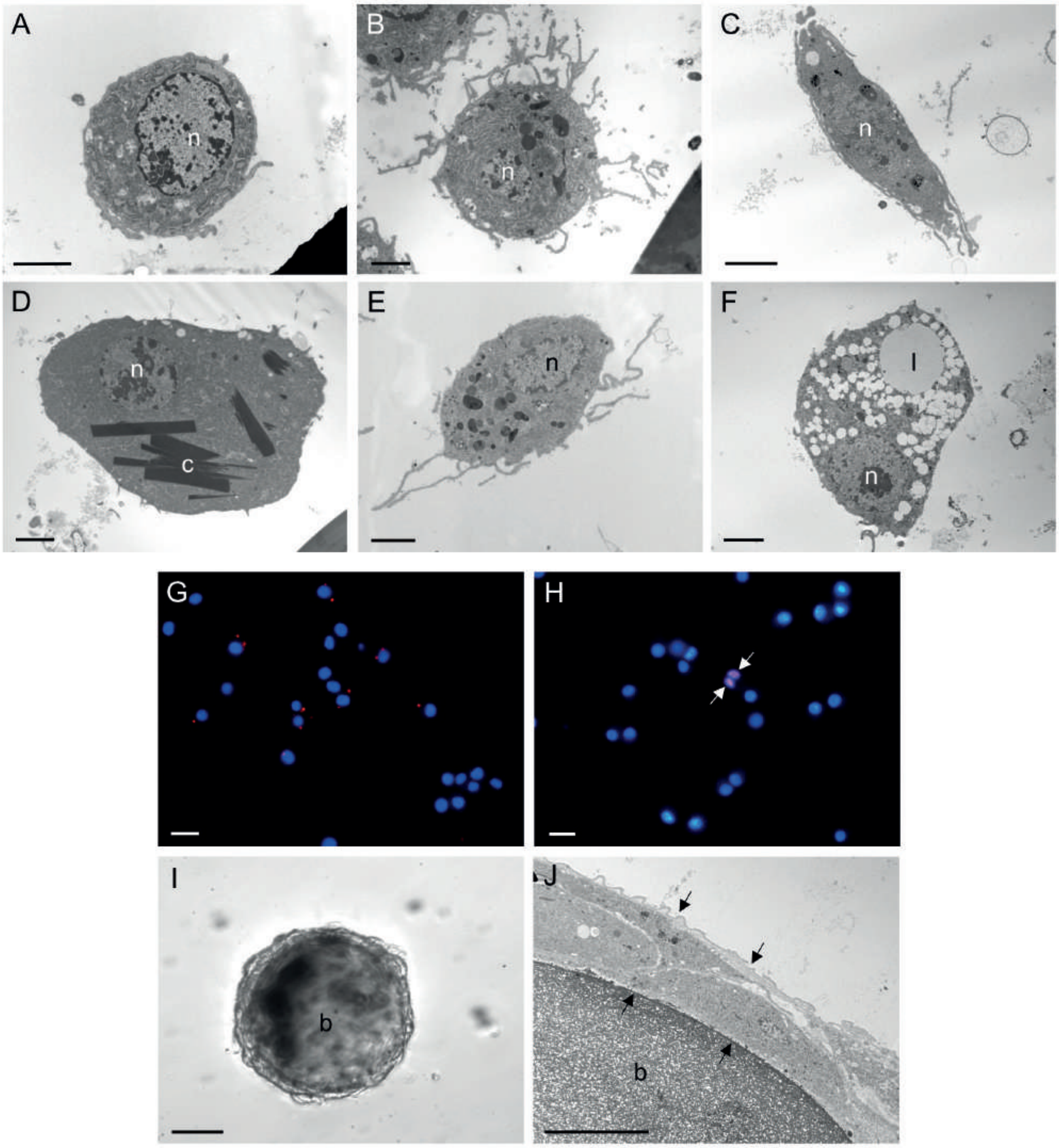

Fig. 1 - (A-F) Immagini in microscopia elettronica a trasmissione (TEM) delle diverse tipologie di emociti di Hermetia illucens: proemocita (A); plasmatocita (B); lamellocita (C); crystal cell (D); granulocita (E); adipoemocita (F). (G) Emociti impegnati nella fagocitosi di batteri fluorescenti (segnale rosso). (H). Emociti in fase di proliferazione (frecce). (I) Incapsulamento di una bead cromatografica di Sephadex (b) da parte dagli emociti. (J) Dettaglio al TEM di emociti (frecce) stratificati su una bead di Sephadex (b) durante il processo di incapsulamento. c: inclusioni cristalline; l: inclusioni lipidiche; n: nucleo.

Scale bar: 2 μm (A-F); 10 μm (G, H); 40 μm (I); 5 μm (J)

Questo meccanismo di protezione sembra necessario per impedire la produzione di melanina, se non strettamente necessaria, in quanto questo pigmento può risultare tossico per la larva stessa.

Un aspetto interessante, che necessita di ulteriori indagini, è che la risposta immunitaria di *H. illucens* è supportata dagli adipoemociti, una particolare tipologia di emociti, finora poco caratterizzata negli insetti, che sembra essere deputata a sostenere dal punto di vista trofico le altre popolazioni emocitarie durante la loro azione.

Riassumendo, i dati ottenuti dalle indagini condotte dimostrano che:

- la risposta cellulare e umorale nelle larve di *H. illu-*

cens mostrano cinetiche diverse, con l'attivazione precoce di fagocitosi e incapsulamento dopo l'infezione batterica, seguito dall'intervento tardivo della componente umorale;

- nonostante i batteri Gram-positivi e Gram-negativi siano rimossi completamente dal corpo dell'insetto in poche ore dall'infezione, grazie all'azione combinata degli emociti e dei fattori umorali, i primi risultano avere una maggiore persistenza nell'emolinfa;

- le prime fasi della risposta cellulo-mediata coinvolgono diversi tipi di emociti che collaborano in una serie coordinata di processi (si veda, ad esempio, la risposta di incapsulamento).

Queste evidenze rappresentano importanti spunti per sviluppi futuri: i) tali informazioni ampliano le conoscenze sui sistemi di difesa di *H. illucens*, un aspetto praticamente sconosciuto fino a pochi anni fa; ii) questi studi costituiscono la base di partenza per intraprendere studi successivi sulla risposta delle larve di *H. illucens* all'attacco da parte di altri patogeni e agenti infettivi, quali funghi o virus; iii) una volta che questi dati saranno completati da informazioni sul ruolo dell'intestino nella difesa dell'insetto dai patogeni, si avrà la possibilità di indagare meglio la dinamica di questi agenti estranei all'interno della larva e di acquisire informazioni preziose sulla sicurezza degli insetti utilizzati a scopo alimentare; iv) tali risultati rappresentano un prerequisito fondamentale per manipolare la risposta immunitaria delle larve tramite fattori ambientali o nutrizionali, al fine di aumentare la loro resistenza ai patogeni, nonché di ottimizzare il loro stato di salute in condizioni di allevamento massale. A tal proposito, dati ottenuti nel nostro laboratorio e recenti pubblicazioni (Vogel *et al.*, 2018; Candian *et al.*, 2022; Candian *et al.*, 2023) mostrano come l'attività antimicrobica della larva e l'espressione di AMPs possano essere modulati dal substrato alimentare fornito all'insetto. Questi risultati aprono promettenti prospettive per quanto riguarda una possibile manipolazione della risposta immunitaria di *H. illucens* attraverso la dieta, con effetti benefici sulle larve.

Riassunto

Le larve di *Hermetia illucens*, nota anche come mosca soldato nera, stanno riscontrando un crescente interesse a livello mondiale per quanto riguarda le applicazioni legate alla bioconversione dei rifiuti organici. Poiché questi insetti vivono all'interno di substrati in decomposizione, potenzialmente ricchi di patogeni che possono compromettere il loro stato di salute, crescita e riproduzione, queste larve hanno sviluppato molto probabilmente un sistema immunitario sofisticato ed efficiente. Questo è un aspetto rilevante, finora poco studiato, che può avere un impatto sull'allevamento massale delle larve negli impianti produttivi e può influenzare la qualità microbiologica della farina prodotta dall'insetto. In questo articolo viene presentata una sintesi delle conoscenze relative alla risposta immunitaria delle larve di questo dittero, concentrando l'attenzione sulle due componenti principali del sistema immunitario, ovvero la risposta cellulare e quella umorale.

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THE FATE OF FOOD PATHOGENS DURING BLACK SOLDIER FLY REARING

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The fate of food pathogens during black soldier fly rearing

The larvae of the black soldier fly (BSFL, *Hermetia illucens*) are among the most studied and used insects, both in academics and industry, for the conversion of a wide range of organic side/waste streams into valuable biomolecules. One major risk in this process is the microbiological load of the envisioned organic streams, which are prone to contain foodborne pathogens like *Salmonella* or *Staphylococcus aureus*. Though literature indicates that these do not impact larval health, it is still necessary to assess whether these food pathogens will enter the food chain when using these insects as feed. The given presentation summarized the latest findings of the research group on insect production and processing in this domain. First, organic residual streams were evaluated to explore which food pathogens are frequently present. Next, challenge tests were executed with *Salmonella* and *S. aureus* to map their dynamics both at the whole larvae level and in the different sections of the larval gut. These revealed a clear difference in the dynamics between both food pathogens on chicken feed. Finally, future research avenues are suggested to explore the mechanisms behind these differences.

KEY WORDS: Food pathogen, microbiological safety, *Salmonella*, *Staphylococcus aureus*, *Hermetia illucens*

INTRODUCTION

The expanding world population presents a major challenge to our food system, which struggles to meet the associated increased production volumes in a sustainable manner. A key element in this regard are the high losses during food production (ALEXANDER *et al.*, 2017) wet mass, protein and energy. The comparison reveals significant differences between these measurements, and the potential for wet mass figures used in previous studies to be misleading. The result suggest that due to cumulative losses, the proportion of global agricultural dry biomass consumed as food is just 6% (9.0% for energy and 7.6% for protein). For this reason, our food system needs to shift from the linear usage of fossil-based products towards a more efficient (re-)usage of available renewable products. It will have to produce renewable biomass that is efficiently transformed into added value bio-products (YISHAI *et al.*, 2016), while wastes are recycled in close loops. Specific edible insect species have the potential to be key players in this transition as they offer (i) an energy-efficient, high-quality protein source for feed/food (VAN HUIS *et al*., 2015; SALOMONE *et al*., 2017) and/or (ii) a sustainable strategy to upcycle various waste/side streams by converting them into biomass (ČIČKOVÁ *et al.*, 2015). Numerous laboratory studies have shown that several fly species are well suited for biodegradation of organic waste, with the house fly (*Musca domestica L.*).

The Black Soldier Fly (BSF, *Hermetia illucens* L., Diptera: *Stratiomyidae*) has become one of the most important species in the world for bioconversion, being reared by multiple companies on an industrial scale at tonnes per week or month. Its larvae are able to convert a wide range of organic waste streams into biomass (ČIČKOVÁ *et al.*, 2015), composed mainly of protein (used in feed), fat and chitin (used as raw material in several industries) (CULLERE *et al.*, 2016; MAURER *et al.*, 2015; NGUYEN *et al.*, 2015). Protein meal from the BSF larvae is authorised in the EU as feed ingredient in pet food, aquaculture, pigs and poultry (IPIFF, 2021). To maximize the potential of the industrial production of edible insects to help reduce the ecological footprint of our food system the focus should always be put on its sustainability. A common tool to address this is a Life Cycle Assessment (LCA), such analyses can indicate how changes in the system can improve its sustainability (VAN ZANTEN *et al.*, 2018). An LCA study executed for BSF revealed that the use of non-utilized organic side streams and the reduction of the most energy-consuming processing steps, e.g. drying, have the biggest impact (SMETANA *et al.*, 2018).

However from a microbiological perspective, such changes do introduce a number of new risks that warrant further examination. On the one hand, many of these non-utilized organic side streams are more prone to contain a broad range of (harmful) microorganisms, hence it is important to obtain a general overview of their micro-

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bial load and composition. On the other hand, a reduction in energy-consuming steps, which typically include heating, could increase the chance of specific micro-organisms surviving into the final end-product and entering the food chain. Of course, specific criteria have to be met to ensure the microbiological safety, but more detailed information on the actual dynamics of foodborne pathogens during BSF rearing would help finetune the processing steps required to ensure the microbiological safety.

Recent advances in our research group IP&pP

To answer these questions, our research group (IP&P) set-out to explore these aspects for black soldier fly rearing and here I presented an overview of these efforts of which many have been published already in separate articles.

Determining the microbiological load of several organic side streams

The microbiological load of several non-utilized (or hardly utilized) side/waste streams was analyzed in a first stage (Gorrens *et al.*, 2021). Selected side/waste streams were chicken litter, overproduced vegetables from an auction, corn meal, grain mix, apple pulp, fruit puree, catering/supermarket/industrial food waste, household food waste, strawberry leaves, and tomato leaves. Zooming in on the food pathogen *Salmonella* only revealed its presence in small amounts in chicken litter and grain mix but only in one replicate in period 1, resulting in an average count of less than 3.2 and 2.2 log CFU/g, respectively. For all other streams, counts for *Salmonella* were below the detection limit of 2.0 log CFU/g (Gorrens *et al.*, 2021). A more concerning picture was obtained for *S. aureus*. This pathogen was observed in all replicates of all waste streams, with the exception of fruit puree. The highest values (from 7.1 to 8.7 log CFU/g) were measured for chicken litter. However catering/supermarket/industrial food waste also showed high values of 5.5 to 5.6 log CFU/g in both sampling periods. This indicates that both pathogens could enter a BSF rearing facility in theory, though the risk seems higher for *S. aureus*.

Determining the in vivo dynamics of both pathogens through challenge tests

Therefore our group explored how both pathogens behave during rearing when they are effectively present in the substrate, chicken feed, for the larvae. The detailed results of both challenge tests can be found in the following open-access publications for each of the pathogens: *Salmonella* (De Smet *et al.*, 2021) and *S. aureus* (Gorrens *et al.*, 2021). Interestingly the dynamics of both food pathogens in the same substrate differed significantly. In the absence of BSF larvae both pathogens were able to persist in the substrate during the six day long challenge test, however *S. aureus* levels did show a small decrease compared to *Salmonella* levels. In the presence of BSF larvae the *Salmonella* levels remained high until six days after an inoculation with a high inoculation level (7 log CFU/g) in both larvae and substrate. The level of *S. aureus* on the other hand dropped below the detection limit (2 log CFU/g) at the same time point and with the same inoculation level. These observations clearly demonstrate the caution that has to be taken into account with claiming a broad antimicrobial activity of the BSF larvae. Although previous studies did show a decrease in Salmonella levels during BSF rearing on manure (Lalander *et al.*, 2015), such activity seems to vary based on the substrate and the micro-organism.

Zooming in on the dynamics of a gram-positive and -negative bacterium within the BSF gut

As documented by the group of Professor Tettamanti the gut of the BSF larvae can be subdivided in five distinct regions: foregut, anterior midgut, middle midgut, posterior midgut and hindgut (Bonelli *et al.*, 2019). Each of these regions has its own micro-environment (Bonelli *et al.*, 2019). In the final phase of the presentation I summarized the most recent findings of our group in collaboration with Dr. Daniele Bruno from the group of Prof. Tettamanti (University of Insubria). Together we explored how *Escherichia coli* (as a model for gram-negative bacteria) and *S. aureus* (as a gram-positive model) survive the passage through the different gut regions, when different inoculation levels were added to the chicken feed substrate. A more elaborate publication on the matter will be published in the upcoming year, but our preliminary findings confirm the results obtained when observing the whole larvae. Differences in the transit of the two bacteria along the gut were observed. As the middle midgut is characterized by a low pH and high lysozyme activity, this region could likely act as a protective barrier against pathogenic bacteria.

Conclusion

First of all, it is clear a number of potentially harmful micro-organisms will be present in side streams that are valuable for BSF rearing and thus action should be undertaken to monitor their presence throughout the rearing process to avoid unwanted microbial risks in the final products. At the same time, the combination of all these results demonstrate the high level of complexity that determines the dynamics of specific microbes inside the BSF larval gut and/or rearing cycle. While a case can be made that the presence of *S. aureus* seems to pose limited risks, these findings should best be confirmed on the envisioned substrate for industry. Future research should focus on exploring the mechanisms behind the remarkable difference in fate between gram-positive and gram-negative bacteria in the BSF larvae not only observed in the gut in our studies, but in the hemolymph as well (Bruno *et al.*, 2021). Possible explanations could be the lower sensitivity of the latter to the lysozymes in the gut or the presence of specific micro-organisms that aid the larvae in their removal of specific unwanted pathogens, e.g *Trichosporon* spp. isolates that have shown to be active against *S. aureus* (Gorrens *et al.*, 2021).

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REARING OF *TENEBRIO MOLITOR* AND ITS IMPLICATION FOR HUMAN CONSUMPTION

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Rearing of Tenebrio molitor *and its implication for human consumption*

There is increasing interest in alternative protein and, in a circular economy vision, insects represent one of the best sources to exploit. The European Food Safety Authority proposed a list of insect species with the greatest potential to be used as food and feed. Among these, the yellow mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae) is one of the more popular insects considered for this use. Therefore, to make *T. molitor* more competitive with other traditional protein sources, its mass production must be optimized and to achieve this goal is essential the study of biological insect rearing on low-cost food sources. This short review briefly lists the optimal environmental conditions (temperature, moisture, RH), physical status (population density), and dietary conditions for the insect rearing. Moreover, the microbial contamination that might occur in the insect rearing influencing the insect welfare and its implication for human consumption is briefly treated.

Key Words: *Tenebrio molitor*, rearing, welfare, human consumption

Introduction

In recent years, insects have received particular attention as alternative protein sources, overcoming the most criticalities of the conventional sources (Ordoner-Araque and Egas-Montenegro, 2021).

Several studies report that edible insects have a high protein and essential amino acids content, and high digestibility (Payne *et al.*, 2016; Gravel and Doyen, 2019; Orkusz, 2021). Moreover, they are good sources of essential fatty acids, microelements, vitamins, fiber, and bioactive substances (Zielińska *et al.*, 2018).

To date, more than 2000 edible species have been described (Jongema, 2017), but only a few of these have been used as protein sources. The European Food Safety Authority (EFSA, 2015) proposed a list of insect species with the greatest potential to be used as food and feed, which includes the yellow mealworm (YM), *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). Recently, EFSA scientific opinion declared the safety of dried YM larvae (Turck *et al.*, 2020) and of frozen and dried formulations from whole YM larvae (Turk *et al.*, 2021) as a novel food pursuant to Regulation (EU) 2015/2283. Therefore, the YM larvae have a great potential for food production. They represent one of the most promising alternative sources of high-quality proteins, lipids, vitamins, and minerals. Moreover, compared to meat livestock, the feed conversion ratio is higher, the reproduction and growth are faster, the edibility is almost absolute (Errico *et al.*, 2021), and the production requires less land and emits fewer greenhouse gases and ammonia (Oonincx and de Boer, 2012; van Huis and Oonincx, 2017). Above all, YM is relatively easy to culture and needs low water and biotic resources (Le Féon *et al.*, 2019).

Rearing of *tenebrio molitor*

In Europe, the YM feed and food production as a whole larva or as a processed product (protein flours, protein extracts, and oils) has long been conducted on small scale. This rearing method is very easy and cheap. In fact, it allows the use of trays with substrate and all different stages of development together. Although simple, it is highly dependent on manual work. The mechanization of the process is possible (Cortes Ortiz *et al.*, 2016; Oonincx and de Boer 2012), but not competitive yet. To achieve this goal is essential the study of biological insect rearing based on low-cost food sources.

YM shows high plasticity in larval development time, survival rate, larval and pupal weight. The nutritional profile depends on the feeding media (Morales-Ramos *et al.*, 2010; Oonix *et al.*, 2015; van Broekhoven *et al.*, 2015; Dreassi *et al.*, 2017; Francardi *et al.*, 2017; Kim *et al.*, 2017; Morales-Ramos *et al.*, 2019). However, to have a sustainable final product in the shortest time, the right rearing parameters must be first considered. Therefore, the optimal range of environmental conditions, physical status, and dietary rearing conditions must be identified.

The most important environmental parameter is the temperature, on which YM resilience largely depend, even if the interaction of temperature and moisture considera-

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bly affect the larval development and survival. Different stages of YM development required approximately the same temperature (Ribeiro *et al.*, 2018) which is 25–28°C (Punzo and Mutchmor, 1980; Spencer and Spencer, 2006; Koo *et al.*, 2013; Kim *et al.*, 2015), temperature below 17°C inhibits embryonic development (Koo *et al.*, 2013) and temperature at 30°C increases death rates (Ludwig, 1956; Koo *et al.,* 2013). Considering the moisture, with the increase in humidity percentage, the growth rate increases, although this condition could favor the colony contamination by microorganisms and mites (Fraenkel, 1950). Instead, a reduction in humidity percentage could cause temporarily larval starvation (Urs and Hopkins, 1973). Even if less influencing the growth, different values of RH cause variability of YM response. The optimum range of RH is 60% to 75% (Punzo, 1975; Punzo and Mutchmor, 1980; Manojlovic, 1987). A very important aspect to consider is the combination of temperature and RH, cause effects considerably the water absorption capacity in different stages.

Also, particular attention must be given to the population density. Higher population densities result in fewer larval instars and smaller mature larvae size (Connat *et al.*, 1991; Morales-Ramos *et al.,* 2012; Morales-Ramos and Rojas, 2015). Moreover, higher densities inhibit the population, promote the cannibalism (Tschinkel and Willson, 1971), and reduce the growth rates (Weaver and McFarlane, 1990). Another important consequence of the overcrowded populations is the increase in temperature due to the larval metabolism which can be lethal.

Last, but not least, YM is a highly polyphagous insect that do not highlight specific dietary rearing conditions. Different substrates have been studied in the last decade (Ruschioni *et al.*, 2020), showing that YM feeds primarily on farinaceous materials such as flour and meal (Ribeiro *et al.*, 2018), but it can easily grow on low-nutrient vegetables or agri-food industry by-products, crop residues even rich in lignocellulose and other substrates (Ruschioni *et al.*, 2020; Errico *et al.,* 2021). The strong point of YM rearing is that it can consume a lot of by-products, bio-converting them for feed and food production in a circular economy view (Ruschioni *et al.*, 2020). A feature of this insect is the ability to select foods to balance its diet and its ratio intake according to its nutritional needs. Despite this, it has been shown that the growth rate and the production of the colony improve when in the substrate are present: 80-85% of carbohydrates, less than 1% of fats, proteins as yeast and casein and B-complex vitamins. Thus, to have a good larval performance, a supplement of these ingredients is required. YM reared on dry substrates and fed with nutrient resources containing water showed higher growth rates, increasing survival rates, and reduced development time (Oonincx, 2015; Urs and Hopkins, 1973).

Insect welfare and implication for human consumption

As mentioned above, edible insects attracted the attention of the European Union. Among them, YM represents one of the most popular species used for the large-scale conversion of several by-products into protein. Therefore, according to EFSA, a proper risk assessment should be carried out to evaluate the potential rearing risks (EFSA, 2015). In fact, the contamination that might occur in the insect rearing, may influence the insect welfare and its implication for human consumption. Generally, among the safety hazards borne by edible insects, microorganisms are the most noteworthy. YM can be infected by microorganisms occurring in the feed or in the rearing environment that can naturally contaminate the external cuticle of insects as well as their intestines through ingestion of contaminated feed (Osimani *et al.*, 2017; Osimani *et al.*, 2018a, 2018b; Garofalo *et al.*, 2019). Moreover, microbial contamination could be a result of vertical transmission from the mother to the offspring (Osimani *et al.*, 2021). Cesaro *et al.* (2022 a,b). As showed previously optimal rearing environment conditions are not favorable for microbial survival and multiplication. Moreover, the larvae fed with contaminated substrates seemed to be environments for microbial survival or multiplication. These data suggested the low possibility of negative implications for human consumption. The low persistence of the microorganism in the farm reduces the possibility of YM to be infected. This result is very important, since the microorganism infection may influence the insect resistance to abiotic and biotic stress and, consequently, the quality of the production. The presence of microorganisms can lead to an alteration of the intestinal microbiome that plays important roles in YM health and welfare (mobilizing stored nitrogen, providing essential nutrients and amino acids for the host, increasing its immune system), thus affecting the development of the intestinal epithelium, the growth of the organism and the survival (Coon *et al.*, 2014; Ruokolainen *et al.*, 2016the gut microbiota plays a role in digestion and metabolism of the host as well as protects the host against pathogens. In the study reported here, we sampled gut microbiota of the larvae of the Glanville fritillary butterfly (Melitaea cinxia; Engel and Moran, 2013). The intestinal microbiome alteration, may lead to a reduction in growth, consequently compromising the production. The investigation of different hazardous microorganism effects on the growth and the survival of YM is essential to reach a high level of quality in rearing. Recent studies showed that YM performance and the mid intestinal conditions are not influenced by target microorganisms (Lepre *et al.*, in progress). Further research is needed to better understand the relationship between microbe contamination and YM rearing.

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WASTE RECYLING WITH FLY LARVAE: FROM SCIENCE TO PRACTICE

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Waste recyling with fly larvae: from science to practice

The black soldier fly (BSF), *Hermetia illucens*, is of the dipteran family Stratiomyidae. It can be encountered in nature worldwide in the tropical and sub-tropical areas. Black soldier fly larvae (BSFL) treatment is an emerging technology for the conversion of biowaste using the natural capacity of the immature life stage of BSF to grow on a wide variety of organic materials. Unknown or variable performance for different biowastes is currently one challenge that prohibits the global up-scaling of this technology with potential positive environmental and societal impacts. This article presents three applied research projects working towards reliable and efficient biowaste treatment with BSFL on heterogenous feedstocks to produce feed and fertilizer products. (1) Estimating feedstock nutrient value from in vitro digestions, (2) balancing feedstock nutrient composition with feedstock formulation and (3) smart process control to respond to variable feedstock compositions. There is an enormous research gap on many aspects regarding this insect that different disciplines of entomology can address (e.g. physiology, nutrient absorption, growth signaling, genetics, reproduction, behavior).

Key Words: black soldier fly, diptera, *hermetia illucens*, circular economy

Black soldier fly biowaste treatment

The black soldier fly (BSF), *Hermetia illucens*, is of the dipteran family Stratiomyidae. It can be encountered in nature worldwide in the tropical and sub-tropical areas between the latitudes of 40°S and 45°N (Dortmans *et al.*, 2017). Black soldier fly larvae (BSFL) treatment is an emerging technology for the conversion of biowaste using the natural capacity of the immature life stage of BSF to grow on a wide variety of organic materials. BSFL treatment converts biowastes and agri-food byproducts into marketable high-value products according to circular economy principles (Bortolini *et al.*, 2020; Cappellozza *et al.*, 2019; Vilcinskas, 2013; Wang & Shelomi, 2017).

In addition to the recycling of nutrients into raw materials for fertilizer, lubricants and biodiesel, pharmaceuticals, and animal feeds markets, BSFL treatment can have reduce environmental impact. For example, depending on their operational conditions, BSFL conversion can reduce emissions from biowaste treatment in comparison to composting (Ermolaev *et al.*, 2019; Mertenat *et al.*, 2019; Pang *et al.*, 2020), and animal feed products can have a lower environmental impact than conventional feeds when produced with BSFL (Smetana *et al.*, 2016; 2019).

There is an enormous research gap on many aspects regarding this insect that different disciplines of entomology can address.

From science to practice

BSFL have a high plasticity to different feedstock nutrient compositions. This is a strength because the can grow on almost any non-lignocellulosic biowaste and side streams (*e.g.* manures, slaughterhouse wastes, food waste, agri-food byproducts, sludges). However, BSFL have a variable rearing performance on these feedstocks based on nutrient composition, rearing parameters, microbial communities, genetics and unknown factors. Unknown or variable performance for different biowastes is currently one challenge that prohibits the global up-scaling of this technology with potential positive environmental and societal impacts. This article presents three applied research projects working towards reliable and efficient biowaste treatment with BSFL on heterogenous feedstock to produce feed and fertilizer products.

Estimating feedstock nutrient value from in vitro digestions

One can only influence/control what one can measure. Therefore, this study describes simulated midgut digestion for BSFL to estimate biowaste conversion performance (Gold *et al.,* 2020). Before simulation, the unknown biowaste residence time in the three midgut regions was determined on three diets varying in protein and non-fiber carbohydrate content. For the static *in vitro* model, diet residence times of 15 min, 45 min, and 90 min

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Table 1 - Median of probability density function of the BSFL midgut residence time distributions (min) with three artificial diets varying in protein and NFC content (Gold *et al.*, 2020).

Diets	n*	AMG	MMG	PMG 1	PMG 2	Total
$P^{13}NFC^{8}$	233	14	37	62	36	154
$P^{13}NFC^{47}$	218	17	46	77	44	191
$P^{7}NFC^{47}$	176	41	32	65	37	195

* n = total number of larvae dissected per diet. AMG: Anterior midgut; MMG: Middle midgut; PMG: Posterior midgut; n: number of larvae

were used for the anterior, middle, and posterior midgut region, respectively (see Table 1). The model was validated by comparing the ranking of diets based on *in vitro* digestion products to the ranking found in *in vivo* feeding experiments. Four artificial diets and five biowastes were digested using the model, and diet digestibility and supernatant nutrient contents were determined. This approach was able to distinguish broadly the worst and best performing rearing diets. However, for some of the diets, the performance estimated based on *in vitro* results did not match with the results of the feeding experiments. Future studies should try to establish a stronger correlation by considering fly larvae nutrient requirements, hemicellulose digestion, and the diet/gut microbiota. *In vitro* digestion models could be a powerful tool for academia and industry to increase conversion performance of biowastes with BSFL.

Balancing feedstock nutrient composition with feedstock formulation

This research assessed the co-conversion of several feedstock to compensate for variability in the composition of biowastes (Gold *et al.,* 2020). Using detailed nutrient analyses, this research assessed whether mixing biowastes to similar protein and non-fibre carbohydrate (NFC) contents increased the performance and reduced the variability of BSFL treatment in comparison to the treatment of individual wastes. The biowastes examined were mill by-products, human faeces, poultry slaughterhouse waste, cow manure, and canteen waste. Biowaste formulations had a protein-to-NFC ratio of 1:1, a protein content of 14–19%, and a NFC content of 13–15% (dry mass). Performance parameters that were assessed included survival and bioconversion rate, waste reduction, and waste conversion and protein conversion efficiency.

In comparison to poultry feed (benchmark), vegetable canteen waste showed the best performance and cow manure performed worst. Formulations showed significantly improved performance and lower variability in comparison to the individual wastes (see Fig. 1). However, variability in performance was higher than expected for the formulations. One reason for this variability could be different fibre and lipid contents, which correlated with the performance results of the formulations. Overall, this research provides baseline knowledge and guidance on how BSFL treatment facilities may systematically operate using biowastes of varying types and compositions. This knowledge was applied with a company at a BSFL treatment facility in Nairobi, Kenya (Gold *et al.,* 2021).

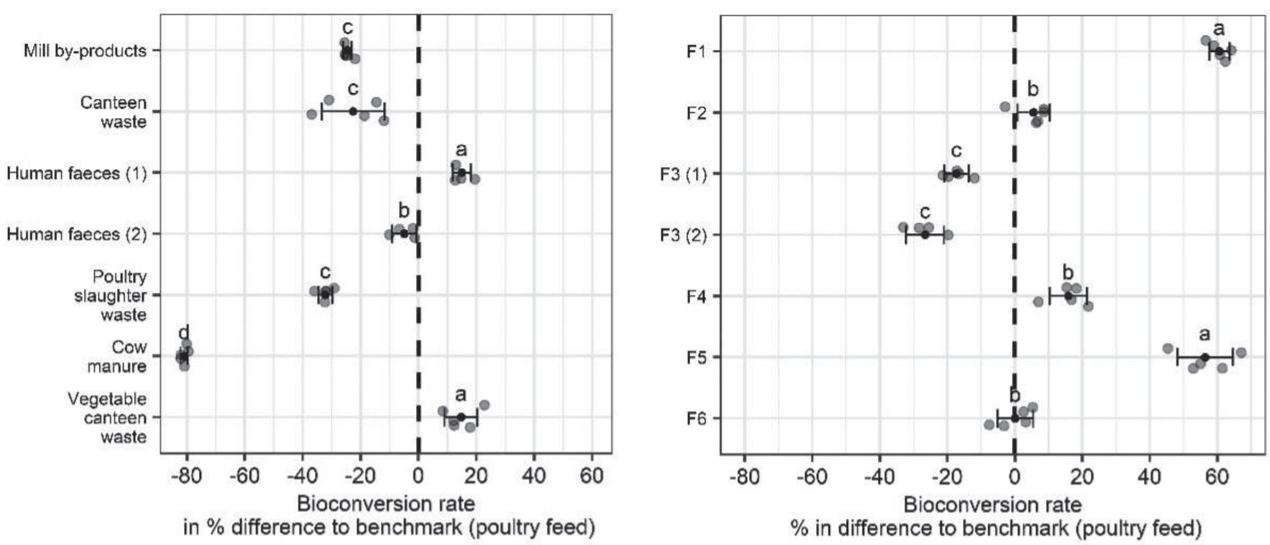

Fig. 1 - Effects of the different waste formulations on bioconversion rate (right) in comparison to the raw feedstocks. Means, standard deviations and results per replicate are displayed (Gold *et al.*, 2020). Performance results with no shared letter are significantly different from each other. All results are given in dry mass.

Smart process control to respond to variable feedstock compositions

Next to measuring the feedstock nutrient value (*i.e. in vitro* formulations), balancing feedstock nutrients (i.e. with formulation based on nutrient composition analyses and larval nutrient requirements) smart process control that responds to the variable rearing performance is a promising approach. Such process control would be especially relevant for smaller sized BSFL treatment facilities, *e.g.* in dense urban environments (e.g. Singapore) where central large-footprint BSFL treatments are not feasible. Process control could be built around real-time measurements of parameters that predict rearing performance. This would be similar to the utilization of other insects, such as bees, where sensors are used to assess colony health. Examples include food waste spectroscopic profiles (*e.g.* near infrared spectroscopy) and real time analysis of growing crate temperature heat maps, larval movement (e.g. using bioacoustics) and even emissions/odors (e.g. H2O, CO2, NH4, CH4). Once relationships are established, these parameters can be measured to vary rearing parameters (*e.g.* ventilation rate, moisture content and pH) with variable biowaste feedstocks.

Key takeways

Research on edible insects including the BSF is highly relevant for industry. Further, practice can deliver relevant research questions that once answered can contribute to positive environmental and societal impacts. There is an enormous research gap on many aspects regarding this insect that different disciplines of entomology can address (*e.g.* physiology, nutrient absorption, growth signaling, genetics, reproduction, behavior). ven though there is competition among insect industry players, companies are frequently interested to pool knowledge if they address the entire industry and are too comprehensive for one company to address (*e.g.* inbreeding, safety, diseases, physiology). As companies mature and establish competent research departments, they can be quality research papers. This collaboration however also must consider the publication culture of research that aims to provide critical and public knowledge.

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INSECTS AS TOOLS FOR MAKING CIRCULAR ECONOMY IN APPLIED RESEARCH PROJECTS

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Insects as tools for making circular economy in applied research projects

The world is currently experiencing a great contradiction between the need to feed a constantly growing human population, satisfying the need for proteins without negatively affecting the environment to obtain them, and on the other hand a shameful food waste along the different levels of the value chain. In this context, insects can play a fundamental role as bioconverters to upcycle the bio-waste into high value-added products (proteins, fats, chitin and frass fertilizer) useful for various industrial and agricultural sectors, starting from the food and feed industry.

In particular, the black soldier fly (BSF) *Hermetia illucens* is the most widely used mini-livestock due to its extraordinarily efficient bioconversion capacity, making it a perfect tool to implement circular economy. BSF has been chosen for the valorization of various types of organic side streams in applied research projects carried out between 2016 and 2023 in Emilia Romagna region, where the agri-food production is a key sector of the region's economy. Project outcomes include: optimization of BSF larval rearing in various agri-food by-products; protocols for the fractionation of BSF biomass; pilot plants for the rearing, processing and stabilization of BSF; a patent for an egg-laying device; agronomic assessments of the frass; development of bird and fish feed formulations; LCA of the different rearing processes of the BSF; development of bioplastics from BSF proteins; GIS-based multi-criteria site suitability assessment for insect farms. Despite the great interest, insect farming is not yet a reality in Italy. Potential reasons for this situation are discussed.

Key Words: Insects as bioconverters, black soldier fly, insect farming, bio-waste valorization

Contradictory issues: growing population, protein hunger and food waste

On November 15, 2022, the human population in the world reached 8 billion units and is projected to reach 9.7 billion by 2050 (Gu *et al.,* 2021). One of the major challenges is to feed the increasing global population with nutritious food with the aim of reducing as much as possible the environmental impact of food production. According to FAO estimates, the world demand for animal-derived protein is expected to rise by 70–80% (FAO, 2017) by 2050. However, the animal-based food supply chain is the one with the greatest environmental impact in terms of GHG (green house gas) emissions, land and water use, acidification and eutrophication potential (Poore & Nemecek, 2018). Furthermore, the current farming systems, both for animal and plant based foods, are the least resilient to all types of hazards, including biotic, abiotic and international risk factors (Tzachor *et al.,* 2021).

On the other side, about one third to one fourth of all food produced for human consumption is lost or wasted globally each year, with a consequent major squandering of water, land, energy, labour and capital (FAO, 2019). In European countries, the total bio-waste (i.e. biodegradable waste, which does not include forestry or agricultural residues, manure, sewage sludge, and waste from the textile and paper industries) in municipal waste is on average 168 kg/person, accounting for 34% of the total waste production (European Environment Agency *et al.*, 2020). Food is lost or wasted throughout the entire supply chain, but mostly in households (53%), followed by processing (19%), food service (12%), food production (11%) and wholesale and retails (5%) (Stenmarck *et al.,* 2016). According to the EU food waste hierarchy, waste prevention has the highest priority, followed by re-use (redistribution to people and use as animal feed), recycling by anaerobic digestion and composting, and by incineration with energy recovery while disposal without energy recovery and in landfills are the least desirable options (European Environment Agency *et al.,* 2020).

Insect farming as a sustainable solution for the contradictory issues

In this context, the challenge is to find sustainable alternatives that upcycle food side streams while providing

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valuable resources to be used for feed and food production. This is where insects come into play. Growing food waste represents a serious environmental, political and social issue and the use of insects as bioconverters of food side streams is a valuable solution to reduce this issue while offering high quality nutrients that can be exploited not only for feed and food production (FAO, 2021) but also for many other industrial and agricultural purposes.

Of the 1,024,000 described insect species (Stork, 2018), 2111 are currently eaten by humans and pets or farm animals (FAO, 2021). Edible insect species are a nutritious alternative due to the macro- and micronutrients they possess. In general, they have a protein content that varies from 40 to 65% (dry matter) depending on the species, they are rich in essential amino acids, mono- and polyunsaturated fatty acids, vitamins, minerals and fiber (chitin) (Ordoñez-Araque *et al.,* 2022). Most of the edible species are harvested from the wild and eaten in tropical-subtropical regions, and there are now only a few species with optimal characteristics for mass rearing in industrial conditions (van Huis, 2020). Currently, the black soldier fly (BSF) *Hermetia illucens* (Diptera, Stratiomyidae) (Fig. 1) is the most widely used edible insect as mini-livestock globally (van Huis 2020), as the larvae can thrive on a variety of wet organic side streams, including manure and catering waste.

Fig. 1 - The black soldier fly *Hermetia illucens*: mature larvae, adult and eggmasses (Foto Giulia Pinotti)

The bioconversion process carried out by BSF larvae is summarized in Fig. 2. Low-value heterogeneous bio-waste is converted into highly homogenous products with high added value: mature larvae and frass. Larval biomass is rich in proteins, lipids, chitin and antimicrobial bioactive compounds, substances which are used in the feed/food industry and in many other industrial contexts (biomedical uses, cosmetics, biodiesel, bioplastics, biodegradable/edible packaging, emulsifying and foaming agents, lubricants) (Müller *et al.,* 2017, Ravi *et al*., 2020, Surendra *et al*., 2020). Larval frass, rich in nutrients and chitin, is an excellent fertilizer for agricultural purposes (Kaczor *et al.,* 2022, Lopes *et al*., 2022).

The average larval development time is typically 15-20 days and the average reduction of the initial substrate is 60-80%, but these parameters vary greatly according to the rearing substrate; besides, due to the larval activity there is a significant decline in the bacterial load and bad smells (Beskin *et al*. 2018, Gold *et al.* 2018).

Applied research projects focused on black soldier fly in Emilia Romagna

Due to the extraordinarily efficient bioconversion ability, BSF makes a perfect tool to implement circular economy. For this reason, BSF has been chosen for the valorization of various types of organic side streams in applied research projects (Fig. 3) carried out in Italy between 2016 and 2023 in Emilia Romagna region, where the agri-food production is a key sector of the region's economy. In Emilia Romagna there are many agri-food companies, especially in the food processing chain, with consequent high amounts and large availability of agro-industrial production waste and by-products. A small fraction of these side streams is used for animal feed, composting and energy production. However, most of this bio-waste is either incinerated or sent to landfill and alternatives that allow its upcycling are strongly needed. The projects, whose main features are summarized in Fig. 3, are the following: ValoriBio, Flies4Value, Bioeco-Flies, Flies4Feed and Scalibur. ValoriBio and Flies4Value were regional "research & innovation" pro-

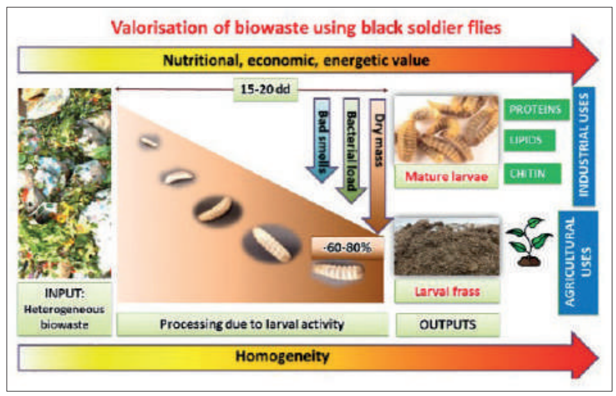

Fig. 2 - Schematization of the bioconversion process carried out by *Hermetia illucens* larvae.

jects whose financed partners were applied research centres located Emilia Romagna. Each project also had to include companies, which had to have an active role in the project (such as donors of materials, potential end users) with staff involvement, but were not financed. Scalibur was an "innovation action" in the Horizon 2020 Research and Innovation Programme, whose partners included research centres, companies and municipalities from several EU countries. The general objectives of this type of projects is to provide innovative research based solutions and technologies (i.e. pilot plants, patents), exploitable by companies and the community, useful for the development of new value chains.

Bioeco-Flies and Flies4Feed are rural development projects whose partners are research centres and farms and the objective is to provide farmers with "ready-to-use" research based solutions, i.e. the diversification of agricultural activities, an alternative source of income.

All projects had specific dissemination plans dedicated to stakeholders and the scientific community, namely: research articles, informative articles for non-experts, press-releases, meetings for stakeholders, field and laboratory visits, training courses for farmers (only for rural development projects). All projects were carried out by teams with highly multidisciplinary skills. All the projects had entomology, food chemistry, LCA, and engineering (for the creation of prototypes) in common. Considering specific expertise, Bioeco-Flies and Flies4Feed had agronomy and animal feed, respectively; ValoriBio had chemometrics, geology, science and material technology, agronomy, food technology and legal studies; Flies4Value had food technology, microbiology, animal feed, agronomy, GIS expertise, sociology; Scalibur had food technology, science and material technology and legal studies.

The prototypes built in ValoriBio (https://www.valoribio.eu/en/) aimed at optimizing BSF larvae rearing on chicken manure, water and chabazite (Bortolini *et al.*, 2020), and the adult conditions to maximize egg laying (Macavei *et al.*, 2020), including the development of a specific device for BSF egglaying, which has been patented (Benassi *et al.*, 2018). Specific protocols have been developed for BSF larvae fractionation, performing also a life cycle analysis (LCA) (Caligiani *et al.*, 2018; 2019, Rosa *et al.*, 2020). BSF proteins have been used to develop a biodegradable bioplastic film (Barbi *et al.*, 2019; 2021) to be used as a mulch sheet (Setti *et al.* 2020), while the frass has been evaluated as a soil conditioner in soilless production of potted baby leaf lettuce, basil and tomato plants (Setti *et al.*, 2019).

The challenge of Bioeco-Flies (https://progetti.crpv.it/Home/ProjectDetail/28) was the valorization of seasonal agrifood by-products to rear BSF larvae year-round (Barbi *et al.*, 2020), assessing also the effect of the rearing substrate on total protein and amino acid composition of BSF larvae (Fuso *et al.*, 2021). The performance of BSF larvae was evaluated in a mass production prototype, the frass was tested on lettuce production and an LCA was performed (Maistrello *et al.* 2020).

In Flies4Value (https://flies4value.it/en) an optimal mix of stabilized agri-food by-products was used to obtain carotenoid-rich BSF larvae (Leni *et al.*, 2022), which were used to formulate a specific feed for laying hens in order to obtain eggs with naturally red yolk. The project also evaluated the acceptability of eggs obtained from insect-fed laying hens by Italian consumers (Lippi *et al.*, 2021) and developed a GIS-based Multi Criteria Decision Making analysis to evaluate the suitability of Emilia Romagna territory to the installation of insect farms (Fiorillo *et al*., 2022).

In the European project Scalibur, the work package lead by UNIMORE was dedicated to obtaining proteins from municipal bio-waste using insects as bioconverters. The ValoriBio prototype for larvae was implemented for the mass rearing of BSF larvae on HO.RE.CA (hotel-restaurant-catering) waste (*i.e.* side streams from a canteen) and a new prototype for the insect fractionation was built. Other groups have evaluated the suitability of BSF larvae meal as an ingredient for dog food and the use of BSF chitin to develop innovative biomaterials for packaging (https://scalibur.eu/resources/).

In Flies4Feed (http://flies4feed.crpa.it/nqcontent.cfm?a_id=22320), still in progress, thanks to the association between an insect farm prototype and a biogas production plant, the excess heat of the latter is exploited to breed BSF larvae on mill waste (Sinisgalli *et al.,* 2022). The BSF meal is then used to formulate specific feeds for trout and pigeons.

Why is insect farming not yet a reality in Italy?

In Europe, the use of insects as tools to upcycle organic side streams into valuable resources, thus putting the circular economy into practice, is severely limited by specific regulations (Reg. (EC) No 999/2001; Reg. (EC) No 767/2009) which prevent the use of manure and any substrate formally recognized as "waste", including domestic and HO.RE.CA food waste, as feed for animals. These regulations apply to any type of farmed animals, and farmed insects are also subject to this rule. The primary concern is related to the safety of the insect meal and frass, in terms of heavy metals and other potentially dangerous contaminants. Although in the laboratory BSF larvae exhibited bioaccumulation of several elements, including metals, from optimal feed sources, a pilot-scale study showed that a former foodstuffs-based mixture resulted in a highly efficient and heavy metal-free production of BSF larvae and frass (Gligorescu *et al.,* 2022).

Project title and logo	Years; role	Funding program	Funding entity	Biowaste used as substrate	Main outputs
GOi Flies4Feed	2021 - 2023 PI	Rural Devel. Plan 2014–2020Op. 16.1.01 – GO EIP-Agri - FA 3A	Emilia Romagna Region	By-products of vegetable origin from agri-food industries	Prototype for BSFL farming associated to biogas plant; optimization of insect rearing; feed formulations for trout and pigeons
BIOECO-FLIES	2017 - 2019 PI	Rural Devel. Plan 2014–2020Op. 16.1.01 – GO EIP-Agri - FA 5C	Emilia Romagna Region	By-products of vegetable origin from agri-food industries	Optimization of BSFL rearing on seasonal substrates, also in a pilot plant; agronomic evaluations of frass on lettuce
FLIES 4 VALUE	2019-2021 Coordinator & PI	Eur. Reg. Dev. Fund (ERDF) Progr. 2014-2020, AXIS 1 Res. & Innovation PG/2018/631984	Emilia Romagna Region	Plant by-products from agri-food industries; by-products from dairy industry	Prototypes for BSFL rearing & stabilization; optimization of BSFL rearing; carotenoid-rich BSFL-based feed for laying hens; eggs with red yolks; GIS-based multi-criteria site suitability assessment for insect farms
ValoriBio	2016-2018 Coordinator & PI	Eur. Reg. Dev. Fund (ERDF) Progr. 2014-2020, AXIS 1 Res. & Innovation PG/2015/737518	Emilia Romagna Region	Poultry manure	Prototypes for insect (A-L) farming; optimization of insect rearing (A-L); patent on an egg-laying device; BSFL fractionation protocols; bioplastics from insect proteins; agronomic evaluations of frass
SCALIBUR	2018-2022 Participant	H2020 Research and Innovation Progr. CE-SFS-25-2018, IA	European Union	HO.RE.CA waste from a canteen	Implemented prototypes for a) insect farming (A-L); b) insect fractionation; optimization of insect rearing; evaluations of BSFL as dog food; BSFL chitin for innovative biomaterials and packaging

Fig. 3 - Applied research projects on the use of *Hermetia illucens* as bioconverter to valorise different types of biowaste. All project were carried out at the Applied Entomology Laboratory, Centro Interdipartimentale BIOGEST-SITEIA, UNIMORE (= Università di Modena e Reggio Emilia) (Role= role of L. Maistrello in the project, BSFL= black soldier fly larvae, A= adults, L= larvae)

In any case, the insect farming sector for feed and food purposes is growing strongly in several European countries. According to the European interest organization for insect producers (IPIFF), the annual production of insect protein in Europe is expected to be 3,000-5000 million kg/year in 2030 (https://ipiff.org/). Yet, in Italy, despite the great interest aroused by the aforementioned projects thanks to specific dissemination events and articles and during other initiatives organized by various bodies, the number of insect farms currently operating is limited to very few units.

The main reasons for this situation can essentially be traced back to a few issues. In the first place, an unclear legislation, mainly related to the subtle difference on what should be considered a by-product or waste (D. lgs. 152/2006 – TUA, art.184 bis; Regulation (EC) n. 1069/2009; Reg. (UE) n. 142/2011). Secondly, economic reasons, namely the need for large initial investments and the absence of a specific incentive system that favours this sector. Thirdly, the lack of specially trained personnel capable of deal with technical problems of insect farming. Finally, the difficulties in finding and connecting the right partners and stakeholders. In this regard, the creation of a specific network to connect all the elements of the new value chain would be highly desirable: from potential investors, to suppliers of suitable bio-waste, to the those who possess the necessary plant technology for insect farming and processing, to those who have the appropriate scientific knowledge and practical experience to carry out insect farming, to potential end users of insect proteins, fats, chitin, antimicrobial compounds and frass in various industrial and agricultural sectors.

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