Effects of feed and probiotic bacteria on *Tenebrio molitor* performance and entomopathogen susceptibility

Carlotta Savio

Propositions

1. Interactions between the host and beneficial symbionts are essential for maintaining host health.

   (this thesis)

2. Provision of a given probiotic decreases the incidence of pathogen infection in mass - reared insects.

   (this thesis)

3. The publication of studies with non-significant findings makes research more efficient.
4. Changing the definition of intelligence to a non-anthropocentric point of view will lead to increased respect for all living organisms.
5. Supporting diversity, equality and inclusiveness in the work environment improves employees' health.
6. The application of artificial intelligence accompanied by the establishment of ethical standards will lead to increased inclusiveness.

Propositions belonging to the thesis entitled:

"Effects of feed and probiotic bacteria on *Tenebrio molitor* performance and entomopathogen susceptibility"

Carlotta Savio
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Chapter 1

General introduction

Entomophagy and the protein production dilemma

The class Insecta is estimated to be around 5.5 million species worldwide, of which only 1 million have been described (Stork, 2018). Their evolutionary plasticity allowed them to survive several climatic challenges since the Cambrian period, occupying narrow niches and assuring ecosystem biodiversity by, among other traits, acting as pollinators and nitrogen recyclers (Shaw, 2014). Dietary habits of ancient human populations involved insect harvesting to complete a diet that was mainly based on cereals, that are poor in essential amino acids, minerals and fatty acids (National Research Council (US) 1989), which then provided an additional protein source and ensured food security (De Carvalho et al., 2020; van Huis, 2013).

First thoughts related to insect consumption in western society have been proposed by Vincent Holt (1885) with the book *Why not eat insects?*. The presence of crop pest as *Pontia brassicae* Hübner (Lepidoptera: Pieridae) and the palm weevil (*Rhynchophorus palmarum* L.; Coleoptera: Dryophtoridae), could be mitigated by adopting the species in the human diet, obtaining a double goal with protecting the crops and increasing the diet quality for people with low income.

Entomophagy habits have been acquired in different ways worldwide throughout time. Indications of insect consumption are present in the ancient Greek and Christian culture, where locusts are referred to as delicious food. Insects from the Coleoptera order have a long history of consumption. Documentation of the ancient roman culture mention the consumption of *Cossus cossus* L. (Lepidopterae: Cossidae), a caterpillar living in the wood of willow trees, the stag beetle (*Lucanus cervus* L.; Coleoptera: Lucanidae) and the palm weevil during their banquets. Other species such as the cockchafer (*Melolontha hypoleuca* F.; Coleoptera: Melolonthinae) were consumed in Java and the yellow mealworm (*Tenebrio molitor* L.; Coleoptera: Tenebrionidae) in Turkey (Holt, 1885). Nowadays, the order Coleoptera comprises 31% of the 2000 edible insect species (Jongema 2019).

The combined factors of physiology, social behavior and nutritional needs and value of some of these insect species determines their application for food and feed production in mass rearing facilities, which is under development in European and North American countries (Hall et al., 2021) along with the already present programs and facilities for insect production used in biological control strategies (Vreysen et al., 2007).

Insect production for food and feed has been taken into consideration for filling the predicted protein gap estimated to develop in the next 30 years (Wang and Shelomi, 2017; Aiking and De Boer, 2020; van Huis, 2020). Along with other protein sources as artificial meat, insects represent a more sustainable solution by demanding less environmental resources for obtaining a comparable amount of conventional protein sources (Henchion et al., 2017; Oonincx and De Boer, 2012; Smetana et al., 2021).

Tenebrio molitor life cycle and circular economy applications

The yellow mealworm *T. molitor* is an European endemic species known mainly as grain pest (Ntalli et al., 2021) commonly used as biological model along with other coleopteran species for studying microbiological and biological interactions (Jo et al., 2021; De Souza et al., 2015).

The adults are dark brown beetles, not able to fly, with dimensions ranging between 13-17 mm (McConnell and Judge, 2018). After sexual maturation, adults can mate and oviposit 500-600 eggs/female, from which larvae hatch after 5-10 days in optimal conditions, T = 26/28°C, RH = 60%, 24 h in the dark (Van Broekhoven et al., 2015). The number of larval stages ranges between 8-20 instars depending on the environmental conditions and on the feed provided (Ludwig, 1956; Morales-Ramos et al., 2010; Scharf et al., 2015). Transition in between each stage is observable by molting of the cuticle, resulting in whiter and softer color of the surface. Larvae then turn into exarate pupae (Scharf et al., 2015). At this stage, the sex of the individual is distinguishable by observing the last segments of the abdomen and the end of the body (Bhattacharya et al., 1970)(Fig. 1 and 2). Adult emergence occurs after 5-10 days and the sexual maturation is reached in the next days (Scharf et al., 2015).

The yellow mealworm production design allows the reallocation of resources by creating a circular system in which plant based byproducts and wastes are used for upgrading the organic matter to high quality nutrients (Van Huis et al., 2021; Van Peer et al., 2021; Yang et al., 2018). Breweries, grain, pulses and legume production sites are sources of ingredients for *T. molitor* feed formulation (Montalbán et al., 2022).

Risk and safety assessment for animals and humans along the food chain is needed when considering the rearing of the insects on wastes and animal byproducts to prevent transmission of human pathogens and prions (Mancini et al., 2019; Van Raamsdonk et al., 2017; Yang et al., 2018; Hardy et al., 2015; Van Der Fels-Klerx et al., 2018).

From processing the yellow mealworm larvae, it is possible to obtain valuable proteins and lipids which can be applied for human consumption since the approval of the European Food Safety Authority in 2021 (EFSA Panel on Nutrition, Novel Foods and Food Allergens, 2021). Insects find their application in human nutrition by supplying proteins (7-48%), lipids (14-74%) and essential minerals such as Zn and Fe in starch-based diets (Aguilar, 2021; Churchward-Venne et al., 2017; Mwangi et al., 2018). The nutrient composition of the yellow mealworm varies depending on the feed provided, depending on diet protein and lipid content (Fasel et al., 2017; Kröncke and Benning, 2023; Oonincx et al., 2015). Moreover, the possibility to increase mineral concentration of larvae before harvesting is interesting in the production for human and animal nutrition (Finke, 2003).

By applying different technologies, it is possible to separate lipids and chitin used for animal nutrition and cosmetic products (Franco et al., 2021; Tognocchi et al., 2023; Van Huis et al., 2021). Frass, feed leftovers and exuviae are applied in agriculture as fertilizer (Barragán-Fonseca et al., 2022)(Fig. 1).

Implementing regulations and procedures is needed to ensure safety for animal and human consumers and to improve the sustainability in the food production chain.

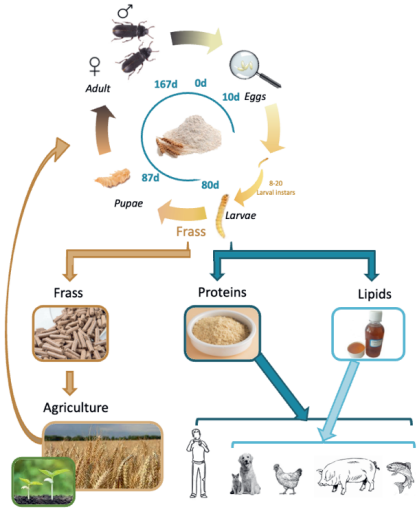

Figure 1. *Tenebrio molitor* life cycle and its application in a circular economy system Agnes Rejasse (GME team, Micalis, INRAE, Jouy en Josas, FR) (inspired by EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) et al., 2021; Van Broekhoven et al., 2015; van Huis, 2013) (d: days).

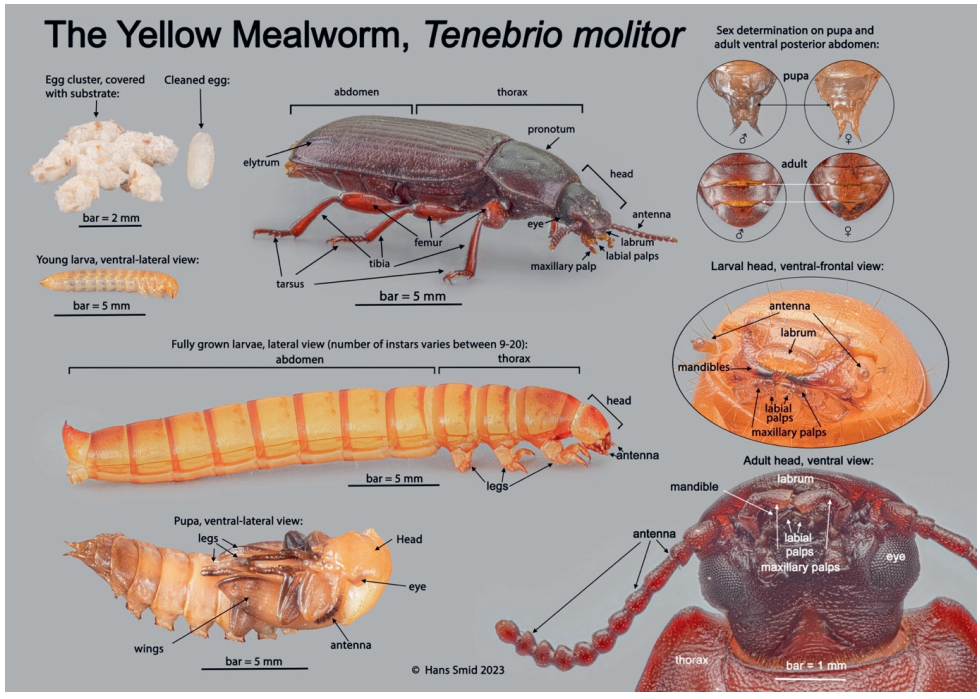

Figure 2. *Tenebrio molitor* developmental chart. The photographs were optimized for increased depth of field by using the photo stacking technique, in order to produce macro photographs in which all details of the insect are in focus. This was done by making multiple images focused at various distances with a camera (Panasonic Dc-G9 mounted on a Stackshot automated macro rail (Cognisys, Traverse city, MI, USA). Resulting stacks of images were processed with Helicon focus vs 8.2.1 software (Heliconsoft, Kharkiv, Ukraine). This software masks the unsharp parts in a stack of images to produce a single photograph of the sharp parts of each image in the stack. Depending on the magnification and size of the insect, between 30 and 150 images were taken to produce one photograph that shows the entire insect in focus (Hans Smid, Laboratory of Entomology, Wageningen University, NL).

Mass-rearing environment

The environment of mass-reared insect is characterized by biotic and abiotic factors that can act as stressors on insect development and health if not optimally regulated (Herren et al., 2023; Maciel-Vergara et al., 2021).

Abiotic factors are environmental parameters such as temperature and relative humidity (Ludwig, 1956; Marshall and Sinclair, 2015; Scharf et al., 2015), and mechanical stressors (Dupriez et al., 2022). Biotic stressors are pathogenic microorganisms such as bacteria, fungi and viruses that can be introduced in the environment by external factors. Employees handling the insects, feed, water and air can be vectors of entomopathogens (Maciel-Vergara et al., 2021; Slowik et al., 2023) that can affect insect health with different levels of incidence. Several infection symptoms have been observed in mass-rearing facilities resulting in lethal or sublethal effects on survival and performance, changes in colour, texture (Fig. 3) and flavor depending on the microorganism responsible for the infection (Slowik et al., 2023).

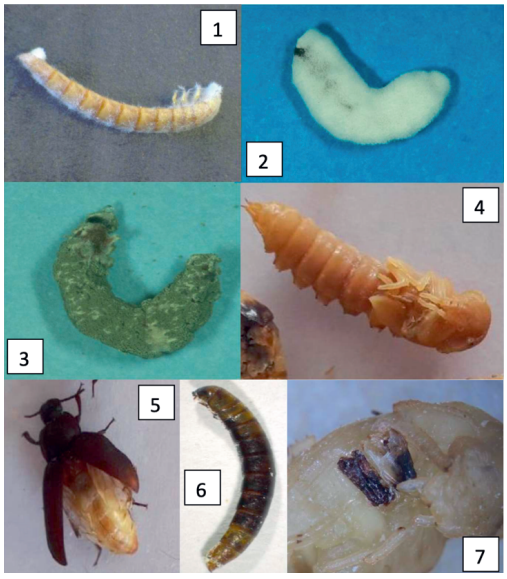

Figure 3. Diseases and disorders in yellow mealworm *Tenebrio molitor* observed by the University of Copenhagen and Entomo Farm (4,5). Symptoms of infection by fungi such as *Beauveria bassiana* (1, 2) *Metarhizium brunneum* (3), bacteria such as *Serratia marcescens* (4) or others (5, 6) and effects of mechanical injury (7). Output from the Insect Doctors consortium (Lecocq et al., 2019).

Good manufacturing practices (GMP) and Hazard Analysis and Critical Control Point (HACCP) guidelines have been adopted and applied for reducing entomopathogen and human pathogen incidence in mass-rearing facilities (IPIFF, 2022), but additional strategies such as immune priming (Tetreau et al., 2019), selective breeding (Song et al., 2022) and probiotic provision (Savio et al., 2022) along with the application of next-generation sequencing (NGS) techniques for pathogen detection (Majewski et al., 2022) can be implemented for assuring the safety of the reared insects along with increasing insect performance and health.

In this study, susceptibility and performance of *Tenebrio molitor* larvae have been tested when exposed to two entomopathogens, which can be applied on cereal crops or stored grains in biocontrol strategies, the bacterial strain *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* and the fungal isolate *Metarhizium brunneum* KVL 12-30.

Bacillus thuringiensis sv. *morrisoni* biovar *tenebrionis*

Bacillus thuringiensis is an ubiquitous Gram-positive sporeforming and crystal toxin-producing bacterium that has been used for biocontrol strategies (Schnepf et al., 1998) and for genetic engineering of insect-resistant plants by the introduction of genes encoding the crystal toxins (Sanahuja et al., 2011). The control efficacy against selected species has been proven, but their effects on non-target organisms and the environment has still to be taken into consideration for assuring the safety along the food chain since the *B. thuringiensis* (Bt) strain used in bio-control is part of the *Bacillus cereus* group of which several species and strains are in volved in human gastro-intestinal infections (Belousova et al., 2021).

Close relationships to Bt incidence and agricultural practices have been observed in studies related to the spread of foodborne diseases, finding higher loads of *Bacillus* spp. pathogens in crops cultivated closer to the soil (Bonis et al., 2021).

The infection route is characterized by oral ingestion and action of the virulence factors, mainly represented by the crystal toxins and spores, in the insect gut lumen (Raymond et al., 2010). The gut pH conditions and the presence of trypsin and chymotrypsin proteases determines the activation of the Bt protoxins in the 55 kDa toxin by proteolysis (Adang et al., 2014). The toxin is then able to bind to midgut receptors resulting in the leakage of the epithelium, allowing the spores or outgrown bacteria to access the haemocoel (Guo et al., 2021; Raymond et al., 2010; Zanchi et al., 2020). The Bt strains along with several other strains from the *Bacillus cereus* group produce non-insect specific virulence factors like the metalloproteases InhA1 and InhA3 that might increase the success of infection by escaping the insect innate immune system through degradation of AMPs and deactivation of haemocytes (Dalhammar and Steiner, 1984) or enterotoxins that could play a role in pathogenesis (Kyei-Poku et al., 2007). Moreover, iron bacterial acquisition systems by Bt and *B. cereus* plays a role in host adaptation and infection (Harvie and Ellar, 2005; Daou et al., 2009; Raymond et al., 2010) (Fig. 4).

Symptoms related to Bt infection are mainly correlated to reduced feeding behaviour, darker cuticle colour and larval death from septicaemia (Milutinović et al., 2015; Zanchi et al., 2020)(Fig. 5).

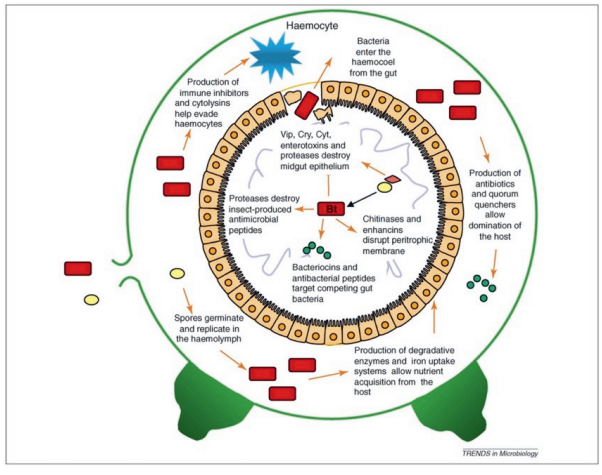

Figure 4. *Bacillus thuringiensis* infection scheme in a transversal section of a lepidopteran insect. The section is seen from the lumen of the central midgut to the peritrophic matrix (interupted grey line), the midgut epithelial cells, the haemocoel and then finally the cuticle (green line). Bt is present in its vegetative form (red rectangle) or in the form of spores (yellow ovals) and crystals (orange) (Raymond et al., 2010).

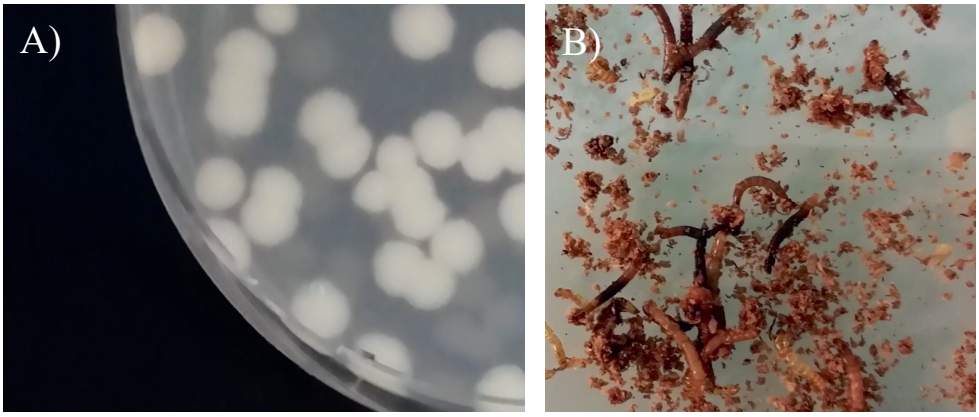

Figure 5. A) *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* grown in ventilated Petri dish containing LBA medium (T = 30°C, 24 h). B). Dead *T. molitor* larvae due to *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* infection. Darker cuticle color and flaccid body consistence are visible after death. Sublethal symptoms are identified in lowered feeding behaviour.

Bacillus thuringiensis sv. *morrisoni* biovar *tenebrionis* (4AA1) (Btt) has genes responsible for the production of Cry3Aa toxin, known for its pathogenic activity against coleopteran insects (Gao et al., 2015; Schnepf et al., 1998). The strain was at first registered as an microbial pesticide to control coleopteran larvae in the US after demonstrating good results in controlling the Colorado potato beetle (Schnepf et al., 1998). Other species from the same order such as *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) presented a susceptibility correlated to the load of ingested spores and higher infection rates increased cannibalistic behaviour (Milutinović et al., 2015) highlighting the importance of the application of GMP in mass-reared insects and the control of the density of the population for reducing the risk of infection in mass-reared insects (IPIFF, 2022; Herren et al., 2023).

Metarhizium brunneum

Entomopathogenic fungi belonging to the genera *Metarhizium* (Metch.) Sorokin (Hypocreales: Clavicipitaceae) are widely distributed pathogens of insects, mites and ticks, playing a role as saprophytes in the natural food chain by digesting cadavers with the excretion of chitinases and proteases (Mantzoukas et al., 2022; Scheepmaker and Butt, 2010; Vilcinskas and Götz, 1999). Some isolates are applied in the environment for biocontrol programs of ticks, mosquitoes and flies, to reduce crop damage and human health risks (Alkhaibari et al., 2016; Bamisile et al., 2021; Behle et al., 2015; Castrillo et al., 2011; Yousef et al., 2013). *Metarhizium spp.* are also applied as biostimulant on several plants of economic interest as oilseed rape (Brassica napus), sitka spruce (Picea sitchensis), maize (Zea mays) and strawberry (Fragaria annanassa), some of which can be used as ingredients of insect feed (Montalbán et al., 2022b; Wood et al., 2022). Meanwhile the biocontrol application dosage (European Parliament, 2009) and GMP (IPIFF, 2022) should assure the absence or low dosages of the conidia in the processed product and in the feed provided to mass-reared insects. The fungal isolate *Metarhizium brunneum* KVL 12-30 was selected for testing *T. molitor* larval susceptibility.

Metarhizium spp. way of infection is characterized by cuticle penetration through proteinase enzyme release and the creation of an appressorium from the germinated conidia (Ment et al., 2020). Moreover, the description of several fungal genes potentially useful for oral infection invites the hypothesis of increased possibility of insect infection by gut penetration after ingestion (Mannino et al., 2019). The infection proceeds with the development of secondary

hyphal bodies and blastospores in the insect hemocel (Fig. 6)(Hajek and St. Leger, 1994). If the insect immune system does not recognize the presence of the pathogen or does not react by humoral or cellular responses, the mycelium composed by the hyphal bodies circulates firstly in the hemolymph, capturing the nutrients and producing toxins (Vilcinskas and Götz, 1999).

During the final phase of fungal development, outgrowth of hyphae is visible from the insect cadaver and conidia are produced in a few days for dispersal in the environment (Fig. 7)(Vilcinskas and Götz, 1999).

The release of secondary metabolites such as destruxin and cytochalasins allow the fungi to impair the cellular recognition and defense reaction of their host, increasing the chance to succeed in the infection process as observed in *Galleria mellonella* infected with *M. anisopliae* (Vilcinskas et al., 1997).

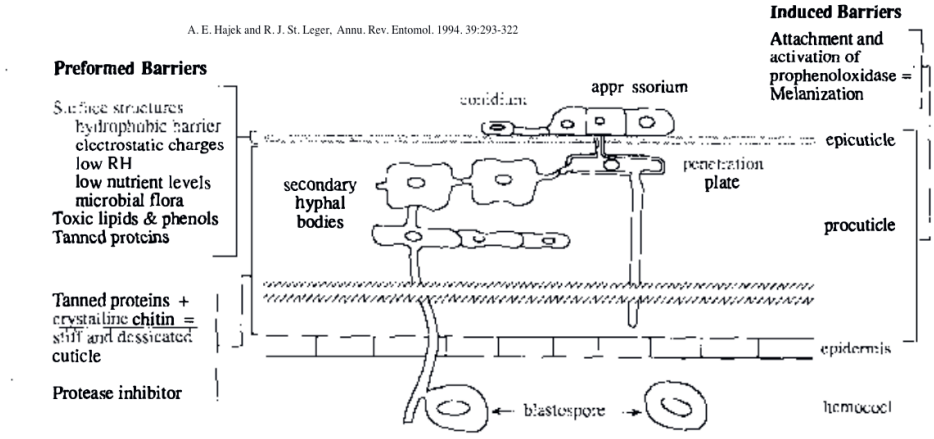

Figure 6. Schematic representation of infection structures of *M. anisopliae* and cuticular resistance barriers in cross–section of insect cuticle (A. E. Hajek and R.J. St. Leger, 1994).

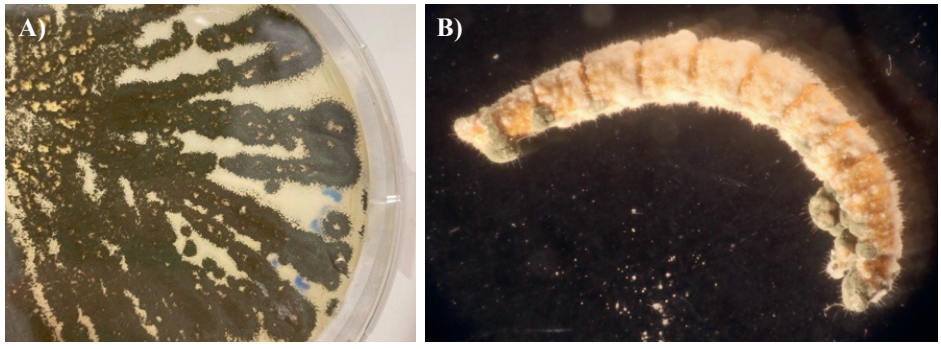

Figure 7. A) *Metarhizium brunneum* KVL 12-30 conidia grown in ventilated Petri dish containing SDA medium (T=23°C, HR=60%, dark conditions, 21 days). B) Yellow mealworm larva infected with *M. brunneum* KVL 12-30. Hyphae (white) and conidia (green) fungal outgrowth is visible after respectively 3 and 5 days after insect death. Pictures made by Christophe Buisson (GME team, Micalis, INRAE, Jouy-en-Josas, FR).

The use of the yellow mealworm for creating entomopathogenic fungal stock/reference libraries for biopesticide production led to the isolation of *M. anisopliae* and *Beauveria bassiana* fungal strains along with other entomopathogenic species (Kim et al., 2018). Despite the high prevalence of the strains, the larval mortality reached 100% and 20% depending on the isolate, raising questions related to insect performance and sublethal effects of fungal infection on host fitness (Eski and Murat Gezgin, 2022; Kim et al., 2018).

Tenebrio molitor larvae presenting higher cuticle melanization levels are associated with lower susceptibility to fungal infection (Armitage and Siva-Jothy, 2005; Barnes and Siva-Jothy, 2000). Darker individuals also display higher levels of phagocytosis. These parameters allow to select for individuals presenting a darker cuticle for reducing the occurrence of fungal infection in rearing system environments.

Beneficial microbes: potential probiotic provision for preserving insect health

The diverse relationships between insects and microbiota have been reviewed by Engel and Moran (2013) and Schmidt and Engel (2021) highlighting the importance of gut microbiota in feed digestion, protection from pathogen infection (Weiss et al., 2019), increased oviposition and fecundity (Gould et al., 2018), enhancing larval performance (Storelli et al., 2011) and in shaping social behaviours (Wong et al., 2017). Microbiota complexity and social habits are also described as directly correlated, finding in beetle species longstanding and dense communities (Schmidt and Engel, 2021).

According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), the provision of a live organism followed by an increased health state of the individuals allows the definition of the microorganism as "probiotic" (Hill et al., 2014).

Probiotic application has been used as preventive treatment for several human and animal diseases such as asthma and food-borne diseases (Chen et al., 2020; Hassan et al., 2022; Meirlaen et al., 2022; Puvanendran et al., 2021). Lactic acid bacteria (LAB) are known for their ability to produce bacteriocins, peroxidase and other enzymes with antimicrobial properties (Heng et al., 2007) and most of them are considered as safe (GRAS - generally recognized as safe) according to the US Food and Drug administration (FDA)(Food and Drug Administration, 2018) and the European Food Safety Authority (EFSA) (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2020). Probiotics have found applications in food and feed domain improving the safety of the products and enhancing the health of the consumers (Ouwehand et al., 2021; Zommiti and Ferchichi, 2021).

Probiotics may act also as performance enhancers and protectors from infections playing as competitors for space (Weiss et al., 2019), nutrients or acting as inhibitors by excreting antimicrobial compounds (Schmidt and Engel, 2021).

Their role in shaping host microbial community and in acting as protection against pathogen infection have been observed in several animals of economic interest such as fish and poultry (Chen et al., 2020; Puvanendran et al., 2021). Investigations of mechanisms related to gut microbiota-host interactions revealed high importance of probiotics in insect oviposition, fecundity, longevity and behaviour (Schmidt and Engel, 2021). The role of probiotics has been studied on some insect species of economic interest with the aim of improving performance and reducing risks of pathogen infections (for a review see Chapter 2 of this thesis).

Questions related to the origin of the selected probiotic species and the range of insect species that could benefit from provision of probiotics in terms of host performance and survival led us to test *Lactobacillus plantarum* WJB, a bacterial species that assured *Drosophila melanogaster* L. (Diptera: Drosophilidae) development in undernutrition conditions (Storelli et al., 2018), on *T. molitor* larvae.

Moreover, the provision of vital and deactivated bacterial species on *T. molitor* performance and pathogen susceptibility were used to investigate whether the vitality of the probiotic is strictly needed for preserving insect health or if postbiotic supplementation could be sufficient for observing positive insect responses (Salminen et al., 2021). In this thesis, we also provided *Pediococcus pentosaceus* KVL B19-01, a possible probiotic isolated from the yellow mealworm gut (Lecocq et al., 2021), and we recorded host responses in order to establish their potential probiotic role in insect performance, pathogen susceptibility, microbial community composition (Chapter 5) and finally impact on the nutritional quality of *T. molitor* larvae

(**Chapter 6**). Their valuable effects on insects infected by entomopathogens potentially present in mass-rearing environments could offer new solutions for preventing and mitigating diseases in mass production (Maciel-Vergara et al., 2021).

Pediococcus pentosaceus

Pediococcus is a genus belonging to the family *Lactobacillaceae*. The cells are spheric cocci bacteria paired in couple or tetrades, Gram-positive and facultative microaerophiles, for which oxygen levels between 3-20% provide optimal growing conditions. *Pediococcus* spp. are homofermentative with carbohydrate assimilation patterns and fermentation that may differ among species and strains (Mora et al., 2006; Papagianni and Anastasiadou, 2009)(Fig. 8).

The species *Pediococcus pentosaceus,* along with others such as *Pediococcus acidilactic,* is an important LAB known for its ability to produce antimicrobial compounds as bacteriocins (Heng et al., 2007; Juturu and Wu, 2018; Todorov et al., 2023), which are active against a broad range of Gram-negative bacteria including human pathogens like *Salmonella typhimurium*, Escherichia coli O157:H7 and Staphylococcus aureus (Enciso-Martínez et al., 2022;Yan et al., 2019). Extracellular compounds responsible for the antimicrobial activities produced by *Pediococcus spp.* are mainly identified in pediocins and biosurfactants (Jiang et al., 2021).

Pediocins are small peptides (<5 kDa) characterized by a -Y-G-N-G-V-N- terminus and the need of a posttranslational enzyme modification at low pH for antimicrobial activity (Papagianni and Anastasiadou, 2009). The applications of *Pediococcus* spp. include its use as starter cultures for meat, vegetable and dairy fermentation, characteristic flavor changes and improved hygienic quality and extended shelf life of the food product by acting as biopreservants (Stiles, 1996).

The strain has been selected for this study due to its possible bio-preservant properties, which could be applied in the mass-rearing facilities by inoculating the species in the feed, thereby reducing the risk of occurrence of human and insect pathogens (Stiles, 1996; Todorov et al., 2023) and improving animal growth abilities (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) et al., 2020; Jiang et al., 2021). Moreover, the isolate applied for this study is originally part of *T. molitor* gut microbiota and its probiotic properties has been already proven in terms of improved insect growth and performance (Lecocq et al., 2021).

Figure 8. *Pediococcus pentosaceus* KVL B19-01 culture in anaerobic conditions on MRS (de Man, Rogosa and Sharpe) broth and agar medium.

Lactobacillus plantarum

Lactobacillus spp. are Gram-positive non-sporulating cells part of the *Lactobacillaceae* family that appear as bacillus with dimension ranging between 3-10 µm (Seddik et al., 2017)(Fig. 9).

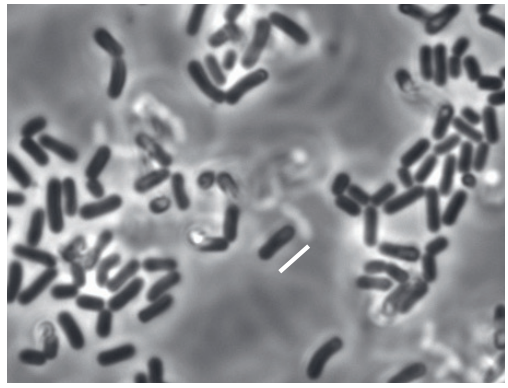

Figure 9. *Lactobacillus plantarum* WJB (light microscopic observation; magnification x100, bar = 3 µm)

Metabolic and adaptation characteristics confer *Lactobacillus plantarum* the ability to colonise several ecological niches such as fermented food products (Berbegal et al., 2016; Jose et al., 2015; Min Hsiu et al., 2016; Valan Arasu et al., 2015) and gastrointestinal, vaginal, and urogenital tracts (Ahrné et al., 1998; Jose et al., 2015; Nami et al., 2014).

Antimicrobial activity against several human and animal pathogens such as *Staphylococcus aureus, Bacillus cereus*, *E. coli* O157:H7, *Salmonella enterica* and *Cronobacter sakazakii* has been recorded due to their ability to produce biosurfactant and extracellular molecules as antimicrobial peptides (Lin and Pan, 2019; Palaniyandi et al., 2017; Yan et al., 2019)(Fig. 10). The pediocine-like bacteriocines have been widely detected in *Lb. plantarum* promoting the species application for increasing food safety (Gupta and Srivastava, 2014; Seddik et al., 2017).

Moreover, *Lb. plantarum*'s ability to produce organic acids such as lactic acid, phenyllactic acid, hydroxyphenyllactic acid, and indole lactic acid lead to antifungal properties that have been exploited as feed preservative (Guimarães et al., 2018; Zivkovic et al., 2019).

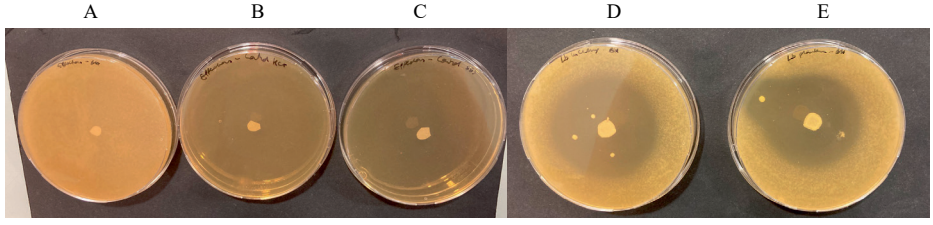

Figure 10. *In vitro* inhibition assay performed on MRS agar and HCT for testing *Lb. plantarum* WJB (D) and *P. pentosaceus* KVL B19-01 (E) isolates' antimicrobial activity against *Bacillus thuringiensis* sv *morrisoni* biovar *tenebrionis* (A) following the method previously applied by Grau et al., (2017). B and C control plates.

Lactobacillus plantarum is available in several probiotic products (Seddik et al., 2017) due to its proven abilities in reducing inflammatory bowel disease, the irritable bowel syndrome and preventing diarrhea.

Application to Diptera and Lepidoptera showed effects of *Lb. plantarum* on host development and performance (Singh et al., 2005; Storelli et al., 2018) raising questions about application to other insect Orders.

Research objectives and outline of this thesis

The purpose of this thesis is to provide a broad overview of *T. molitor* symbionts, conduct experimental studies focused on potential application of beneficial symbionts such as *Pediococcus pentosaceus* KVL B19-01 and *Lactobacillus plantarum* WJB as potential probiotic strains for decreasing the risk of infections in mass-rearing conditions and for optimizing the quality of the chemical composition of the larvae as they may be consumed as feed and food, representing one of the solutions for upscaling the value of by-products along the food chain.

Chapter 2 provides a review of the potential of probiotic applications in mass-reared insects. Methods of isolation, identification and production of probiotic bacterial species are provided, taking into consideration methods of their application in an insect mass-rearing context and the important connections between host-microbiota and provided feed in health preservation and disease management.

Chapter 3, provides a review addressing the richness and the variety of interactions between the yellow mealworm and its symbionts, methods of pathogen detection and insights for reducing the risk of pathogen infection in rearing facilities.

Chapter 4, is an experimental study focused on *T. molitor* susceptibility to the entomopathogen *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis (4AA1)* (Btt) in conditions resembling the mass-rearing system environment. The study highlights the direct correlation between entomopathogen load and the yellow mealworm larval growth and mortality. Larvae having higher body mass showed to be more resistant to the pathogen and were not compromised in feed conversion. The data also showed that Btt can persist in the tested conditions suggesting a certain risk for the rearing.

Chapter 5, focus on the effects of diet composition and two potential probiotic strains, *Pediococcus pentosaceus* KVL B19-01 and *Lactobacillus plantarum* WJB, on performance and microbial community composition of *T. molitor* larvae affected by the entomopathogens *Metarhizium brunneum* KVL 12-30 and *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* (4AA1) single or co-infection.

The larvae presented increased growth and survival in case of co-infection when fed with diet supplemented with *P. pentosaceus*. The bacterial community composition of the larvae was similar in all treatments, indicating that the probiotic strain did not establish at a detectable level in the insect, however, its transient presence improved larval performance in its early developmental stages.

In **Chapter 6**, the effects of diet composition and probiotic provision on *T. molitor* larval performance and chemical composition were studied, through varying carbohydrate-to-protein ratio and either vital or deactivated *P. pentosaceus* KVL B19-01 supplementation. Effects on larval development and on larval body chemical composition, in particular its crude protein and fat contents and the qualitative and semi-quantitative composition of fatty acids.

Chapter 7 presents a general discussion on the experimental results, offering also an overview on the state of art in the field and proposing perspectives for future studies.

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Chapter 2

Bugs in Bugs: the role of probiotics and prebiotics in maintenance of health in mass-reared insects

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Abstract

Interactions between insects and their microbiota affect insect behaviour and evolution. When specific microorganisms are provided as a dietary supplement, insect reproduction, food conversion and growth are enhanced and health is improved in cases of nutritional deficiency or pathogen infection. The purpose of this review is to provide an overview of insect–microbiota interactions, to review the role of probiotics, their general use in insects reared for food and feed, and their interactions with the host microbiota. We review how bacterial strains have been selected for insect species reared for food and feed and discuss methods used to isolate and measure the effectiveness of a probiotic. We outline future perspectives on probiotic applications in mass-reared insects.

Keywords: probiotics; prebiotics; mass-reared insects; insect diseases; microbiota; performance; health

1. Introduction

Insect mass-rearing for food and feed has been identified as a valuable industry with current predictions expecting the market to grow by 47% from 2019 to 2026 ("Edible Insects Market Analysis 2020–2026|Industry Forecasts.," accessed on February 2022). This growth will see production volumes reaching an expected 730,000 tonnes by 2030 (Alliedmarketresearch, 2021). Over 1900 insect species have been reported in literature as being consumed worldwide (van Huis, 2013); however, only a few of these species are mass-reared in a more intensive manner. Much like traditional intensive livestock production, mass-rearing of insects faces similar challenges of high densities, a high rate of pathogen transmission, higher susceptibility to pathogens due to lack of oxygen, high temperatures, and nutrient deficiencies (Cohen, 2018; Maciel-Vergara et al., 2021). These challenges associated with farming insects need to be investigated with urgency in order to develop a successful industry, which has the potential to contribute to global food security.

A solution to mitigate some of these challenges has been the administration of antimicrobials, but this comes with the potential risk of emerging multiple antibiotic resistant 'superbugs' that threaten both animal and human health. The trend has therefore shifted to the use of probiotics which are defined as "live microorganisms that, when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). It is now apparent that every organism is associated with a microbial community ranging from parasitic to mutualistic, and this is true for all animals, from humans to invertebrates, insects included. In particular, the gut microbiota has been of focus to researchers in recent years due to its link to the health status of its host, with more and more studies finding that exploiting these microorganisms may improve animal productivity and maintain their health and wellbeing (Gupta and Nair, 2020; Su et al., 2019).

Interactions between insects and their microbiota play an important role in behaviour and evolution of many insect species (Liberti and Engel, 2020; Richard, 2017). Several microorganisms are able to manipulate host behaviour to increase their transmission. For example, *Wolbachia* which is able to modify the mating preference of its hosts when it acts as a symbiont, and the lack of microbiota or the presence of foreign gut bacteria can distort the feeding behaviour of insects by changing their sense of smell (Liberti and Engel, 2020). Reproduction, conversion, and growth performances have been related to specific microorganisms in mass-reared insects (Jordan and Tomberlin, 2021). Insect–microorganism communication is bi-directional and social interactions in insects can impact the distribution of microorganisms within the population.

Insect diets have, in this context, an important role in providing nutrients both to the insects and the microorganisms. Within its composition, it is possible to highlight specific nutrients that act as prebiotics and that have been defined as "selectively fermented ingredients that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health" (Gibson et al., 2017).

Our goal is to provide an overview of the use of probiotics, and in brief, prebiotics, in the rapidly growing industry of insects for food and feed. As previously mentioned, there are over 1900 species of insects consumed worldwide (van Huis, 2013); however, along with some other relevant examples, this review will focus on the important species currently mass reared in this industry, which includes *Acheta domesticus* (L.) (Orthoptera: Grylloidea), *Gryllus bimaculatus* (De Geer) (Orthoptera: Gryllidae), *Hermetia illucens* (L.) (Diptera: Stratiomyidae), *Musca domestica* (L.) (Diptera: Muscidae), *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae), as well as two other insects of economic importance that are mass-reared, *Bombyx mori* (L.) (Lepidoptera: Bombycidae) for their role in sericulture, and *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), for their role as a non-mammalian model for the study of human pathogens.

This review will highlight the relationship between insects and their gut microbiome and discuss the mechanisms by which probiotics may exert their beneficial effects. It also reviews some of the methods that have been used to reduce the occurrence of disease in reared colonies and gives a summary of the probiotics that have been tested in the seven insect species mentioned. Lastly, we highlight some of the techniques used in isolation of probiotics and ways of testing microorganisms for probiotic potential as well as future perspectives on industrial applications.

2. Defining probiotics and prebiotics: the bugs that debug the bugs

Every organism is associated with a microbial community that may promote its health. The identification of probiotics opened the possibility of exploring the health of insects when they are provided with beneficial microorganisms. In particular, the effect of the microorganisms is assessed for improving growth and reproductive performance and for decreasing the occurrence of diseases in stressful rearing conditions. The World Health Organization's internationally endorsed definition of probiotics is "live microorganisms that, when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). This definition, however, is still unclear in some circumstances, thus causing controversy and confusion. A distinction is made between microorganisms given to insects as a supplement and those that are commensal gut microbes that putatively confer health benefits to the insect (Sanders, 2008). The latter are often erroneously termed as probiotics, but this requires that they be isolated and characterized and their subsequent health-promoting effects validated (Sanders, 2008). Other terms that have been used synonymously are direct-fed microbials given in animal diets and live biotherapeutic products that are more pharmaceutical and take the form of drugs rather than food supplements, even though they are intended for the same use. Overall, the scope of probiotic intervention is expanding, leading to various ways in which the product reaches the market and different regulatory requirements, and this comes with various terms/definitions as a result.

The concept of prebiotics came to light when, in the early 1950s, scientists discovered that there was a special growth-promoting factor in human milk that aided the growth of the probiotic *Bifidobacterium bifidus* (Tissier)(György et al., 1954). These components were later named by Gibson and Robertfroid (1995) as prebiotics and defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health". To put it another way, these are the nutrients that feed the probiotic microorganisms enabling their proliferation in the gut (Lakshmi et al., 2013a). These nutrients are mostly fibers, and they include inulin, oligofructose (produced from inulin), and fructooligosaccharides (FOS) synthetically produced from sucrose, as well as galactose-containing and xylose-containing oligosaccharides, resistant starch (RS), pectin, and other fermentable fibers (Hutkins et al., 2016). It was not long before probiotics and prebiotics were conveniently combined in one synergistic pack known as synbiotics (Gibson et al., 2017; Swanson et al., 2020). For the insect mass rearing industry, this highlights the fundamental importance of different types of diet and diet quality for the success of the probiotic application.

3. The crosstalk between the insect and their intestinal microbiota

The ubiquitous nature of gut bacteria and increasing knowledge of their numerous advantages to insect hosts has led to their application as probiotics in the insect mass rearing industry. The use of probiotics is based on the interaction between the host and their gut microbes; hence, understanding the nature of this crosstalk is fundamental to understanding the process. The insect gut microbiota and their collective genomes ('microbiome') have captured the interest of many researchers today, as an 'organ' in itself that plays a core role in influencing key insect

traits (Engel and Moran, 2013). The insect microbiome consists of a large diversity of microorganisms including bacteria (bacteriome), fungi (mycobiome), viruses (virome), and archaea (archaeome), but bacteria are the most abundant and the most studied (Engel and Moran, 2013).

Studies examining this spectrum of symbiotic relationships have pointed to the beneficial effect of a variety of bacteria and yeast species in different insects and thus their application as probiotics. For example, probiotic application of *Klebsiella oxytoca* and an *Enterobacter* strain increased larval growth of *Ceratitis capitata* (Wiedemann) (Diptera: Tephrtidae) used in Sterile Insect Technique (SIT) application (Augustinos et al., 2015) by increasing larval growth, especially due to bacterial synthesis of nutrients and protection against pathogens through the release of some antimicrobial compounds. A similar effect was also observed in transgenic *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) where aseptically reared larvae that otherwise had low pupal weight and poor survival registered a substantial increase in pupal weight and male fitness when inoculated with *Enterobacter cloacae* (Jordan) (Somerville et al., 2019). However, to initiate any form of host–microbe interaction, a series of steps occur beginning with the microbiota acquisition followed by gut colonization or adhesion and progressing to an establishment in the gut and further transmission back to the environment or to new hosts (Sanchez-Contreras and Vlisidou, 2008). It has been shown that insects can acquire their microbiota horizontally from the environment mainly through diet (Dong et al., 2018; Ojha et al., 2017). Dietary habits have been shown to affect both the composition and robustness of gut communities by regulating nutrient availability for the microbes (Zheng et al., 2020). Microbiota are also acquired by social interaction through trophallaxis and coprophagy transplantation as seen in termites and bees, and thirdly, through vertical transmission from parent to offspring via the egg surface, which is exposed to microbes from the ovaries of the mothers (Weiss et al., 2019).

Adherence of the microorganisms to the gut lumen then follows, but the mechanisms vary across the different species, likely due to diversity in the physiology, morphology, and ecology of insects (Paniagua Voirol et al., 2018; Egert et al., 2005). Gut colonization is affected by many different factors, including physicochemical gut conditions, i.e., pH, redox potential, and oxygen content. Gut compartmentalization can affect microbiota distribution presenting increased microbial density from anterior to posterior compartments. Moreover, the presence of enzymes and immunological compounds in the gut and the life history characteristics causes changes in community abundance (Douglas, 2015; Egert et al., 2005). Depending on the insect species, different bacterial communities have to develop strategies that allow them to survive and persist in the harsh conditions of the host such as the highly alkaline guts of lepidopteran species. To illustrate this, an RNA-sequencing study showed that the gut symbiont *E. mundtii* had upregulated pathways for tolerating high alkaline stress during its passage in the gut of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) (Chen et al., 2016; Mazumdar et al., 2020).

After successful colonization, these microbes may participate in many different symbiotic, pathogenic, or vectoring activities within the host (Dillon and Dillon, 2004; Gupta and Nair, 2020). Focusing mainly on the symbiotic or mutualistic roles, microbes play a key role in metabolism by breaking down indigestible plant-derived polysaccharides through microbiota encoded carbohydrate-degrading enzymes in the midgut and hindgut of caterpillars (Lepidoptera), termites (Dictyoptera: Isoptera), honey bees (Hymenoptera), beetles (Coleoptera), crickets (Orthoptera), and other herbivorous insects (Schmidt and Engel, 2021). These enzymes include cellulases, hemicellulases, and pectinases that are generally absent in insects (Gandotra et al., 2018). Remarkably, recent phylogenetic studies have shown that the genes encoding these enzymes in gut microbes have also been encoded in the genomes of some hosts such as the mustard leaf beetle, *Phaedon cochleariae* (Fabricius) (Coleoptera: Chrysomelidae), signifying horizontal gene transfer (Moran et al., 2008; Pauchet and Heckel,

2013). The breakdown of these plant cell wall materials culminates in the formation of short-chain fatty acids that impact both the microbe and host's nutrition and help in maintaining the integrity of the gut barrier (Paniagua Voirol et al., 2018; Schmidt and Engel, 2021). In this way, gut microbes also compensate for the sometimes nutrient-poor diets of their hosts through nutrient provisioning, for instance, *Buchnera aphidicola* (Munson et al.) in pea aphids that provides essential amino acids that are lacking in the insect diet (Douglas, 1998).

Through provisioning of nutrients and aiding digestion, gut symbionts then positively influence the growth and development of hosts, as shown in numerous studies. A study in *Drosophila melanogaster* (Meigen) (Diptera; Drosophilidae), for example, showed that aseptically reared insects had reduced growth and slower development as compared to conventionally reared insects. However, when inoculated with *Acetobacter pomorum* (Sokkollek), a gut commensal, the growth and development was restored to a similar rate as that of conventionally reared insects (Engel and Moran, 2013). It was also observed that the addition of *Leuconostoc* spp. as a probiotic in the diet of the fruit fly *Bactrocera tryoni* (Froggatt) reduces the mean development time from egg to adult (Shuttleworth et al., 2019).

During herbivory, insects also encounter a variety of toxic plant defense chemicals, the detoxification of which is aided by their gut symbionts which enables their success as pests (van den Bosch and Welte, 2017). This type of detoxifying-symbiosis confers resistance not only to plant allelochemicals but also to insecticides (Xia et al., 2018). This is achieved through diverse mechanisms, for example, enzymatic degradation of potential toxic phytochemical compounds as aglycones by gut symbionts of *T. molitor* (Itoh et al., 2018; Genta et al., 2006). Gut symbionts also play a protective role by increasing the host's resistance to pathogens. Several mechanisms come into play here, including the inhibition of infection, as seen in Tsetse fly *Glossina morsitans* Westwood (Diptera: Glossinidae) colonized with the commensal *Kosakonia cowanii Zambiae* which inhibits *Serratia marcescens* and *Trypanosoma* by raising the pH of the gut (Weiss et al., 2019). Gut symbionts also protect the host by nutrient and space competition with invading pathogens, thus edging them out as observed in mammals and also suspected to be the case in insect hosts (Schmidt and Engel, 2021). The microbiota also enhances the epithelial barrier function to prevent systemic infection by pathogens, as seen in mosquitoes (Rodgers et al., 2017) and the stimulation of the host immune system or immune priming (Schmidt and Engel, 2021).

Overall, the host–microbiota relationship is intricate and intriguing but there are still some aspects that are yet to be explored and well understood for instance the influence of abiotic factors on the interaction and how microbiota impact insect population dynamics as well as behaviour manipulation, all of which could be advantageous in the advancement of probiotic use in the insect mass rearing industry.

Probiotics applications as a means of decreasing disease occurrence in mass reared insects

Mass-reared insects are highly susceptible to diseases caused by organisms that belong to different Phyla within bacteria, viruses, fungi, protista, and nematoda (Eilenberg et al., 2015). Finding a method for preventing these diseases and increasing growth and reproductive performances in insects reared for food and feed purposes is thus a key goal of both industries and researchers (Sikorowski and Lawrence, 1994). Maciel-Vergara et al. (2021) presented an overview of good production practices for reducing risks of pathogen occurrences such as daily adherence to good hygiene practices, differential breeding, mechanical pest control, and techniques such as heat shock/thermal therapy, breeding of tolerant strains, biological control, and RNA interference. In this context, probiotics have also been considered an option for decreasing the impact of diseases due to their ability to positively influence host performance and enhance immune responses against pathogens (Maciel-Vergara et al., 2021; Nishida et al., 2017).

The most known probiotic bacteria are Lactic Acid Bacteria (LAB) that present a high immune system activity in humans (World Gastroenterology Organization, 2017). In insects, antibacterial activity and immune regulatory effects have been widely recorded within the *Lactobacillus* genus in the microbiota of silkworm *Bombyx mori* and the honeybee *Apis mellifera* (L.) (Hymenoptera: Apidae) when infected with the pathogen *Pseudomonas aeruginosa* (Gessard) and *Nosema* spp., respectively (Nishida et al., 2017; Vásquez et al., 2012). Observations of *Galleria mellonella* have highlighted the antimicrobial effects of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* on *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Pneumonia aeruginosa* (Barbosa et al., 2005; Jorjão et al., 2018; Ribeiro et al., 2017). Probiotics strains have been tested against *Nosema ceranae* on honeybee alongside small-molecule RNA interference techniques and supplements for decreasing the bee spore load and viability and increasing its survival and performance (Burnham, 2019). At the genus level, *Lactobacillus* and *Bifidobacteria* had an impact on the pathogens, but when *L. rhamnosus* and sucrose were provided to honeybees to decrease the impact of *N. ceranae*, higher mortality rates and lower phenol oxidase production were recorded (Burnham, 2019; Maruščáková et al., 2020; Ptaszyńska et al., 2016). The positive effects of *Lactobacillus* spp. on hosts infected by fungi have been highlighted in studies focused on *Drosophila melanogaster* and *Galleria mellonella*, respectively, infected with diaporthe FY and *C. albicans* (Rossoni et al., 2018; Su et al., 2019). Other genera such as *Enterococcus* have been studied for decreasing the occurrence of bacterial diseases. *E. mundtii* showed positive effects on *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) immune responses against *Bacillus thuringiensis* infections (Grau et al., 2017). As for viral diseases, the activation of insect-specific or generic immune responses by probiotics is still to be clarified (Maciel-Vergara and Ros, 2017). Although endosymbionts do not fall under the definition of probiotics, it is worth mentioning that the main positive results are related to the presence of *Wolbachia* and *Spiroplasma* that increase resistance to viral diseases in *T. molitor* and *G. mellonella* (Jung et al., 2014; Maciel-Vergara and Ros, 2017). It is interesting to note, however, that some bacteria species isolated from shrimps have been shown to have antiviral activity in a plaque assay and demonstrated positive effects on fitness performance in shrimps infected with white spot syndrome virus, which sheds light on some possible techniques that could be used in screening insect microbiota for the same (Kamei et al., 1988; Lakshmi et al., 2013).

Although the use of probiotics in disease management is very promising, it is still hampered by a scarcity of knowledge on insect-pathogen dynamics and the influence of the stressors on production performances and on insects' susceptibility to diseases. This calls for the need to maintain a holistic approach in the general management of mass rearing systems, taking into account environmental factors, diet, microbiota and genetic factors.

4. The most common microorganisms used as probiotics in insects

The most commonly tested probiotics for insects reared for food and feed belong to the genera *Lactobacillus*, *Saccharomyces*, *Streptococcus*, and *Bacillus* which, according to the World Gastroenterology Organization (World Gastroenterology Organization, 2017), are among the seven 'core' microorganisms most often used as probiotics. *Enterococcus* is a largely underrepresented group, especially given that it is a common symbiont of the insect gut and especially in Lepidoptera. This may be in part due to the fact that *Enterococcus* species have both pathogenic and probiotic strains (Al Atya et al., 2015; Gaspar et al., 2009). Nevertheless, an example of its potential use as a probiotic was described by Grau et al. [58] when they isolated an *E. mundtii* strain from the feces of *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae), which showed antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria, and increased survival in *Tribolium castaneum* beetles after infection with *Bacillus thuringiensis*.

Aside from summarizing probiotics tested for insects reared for food and feed, **Table 1** highlights the lack of research into probiotics for crickets. However, the primary disease observed in reared *A. domesticus* populations is caused by a densovirus (AdDNV). An effective approach to reduce the impacts of the virus on *A. domesticus* populations may not be through the use of probiotics but rather through RNA interference technologies. This approach was used by La Fauce and Owens (La Fauce and Owens, 2013) on *A. domesticus* to reduce PmergDNV titres and subsequent mortality from the virus, by feeding the insect with dsRNA specific to the capsid protein by mixing it into their food. Similarly, there has been little work into probiotics for *Acheta domesticus* (L.) (Orthoptera: Gryllidae), another cricket species mass reared in particular for pet food and human consumption. However, more towards the direction of prebiotics, a recent study found the incorporation of Jew weeds, *Comellina sinensis* (L.) Kuntze, into the diet of the cricket usually just fed chicken feed, resulted in an increase in body weight and improved microbial quality (Ng'ang'a et al., 2020).

Table 1. A summary of probiotics tested on insects mass-reared for food and feed with the objective of improving insect performance or fitness against natural pathogens in the mass rearing environment. The table does not include data where bacteria/yeast have been provided to the insect as a probiotic to test its efficacy against a specific human pathogen in vivo, nor does it include insects reared for sterile insect technique programmes.

Insect Species	Probiotics	Effects on Performance and Yield	Ref.
Silkworm	**Bacteria**		
Bombyx mori	***Bifidobacteria***		
	Bifidobacterium bifidum	Found to be an immunomodulating agent (increase in the activity of protease, amylase and invertase); increased raw silk production with fewer cocoons	(Taha et al., 2017)
	Lactobacilli		
	Lactobacillus acidophilus	Stimulated growth factors leading to an increase in the silk yield and to an improvement of the silk harvest	(Suraporn, S., Sangsuk, W., Chanhan, P., & Promma, S., 2015)
	L. casei	Improved larval weight, cocooning ratio, pupation ratio, and economic characters (cocoon weight and size) when larvae were infected with microsporidium *Nosema bombycis*	(Suraporn and Terenius, 2021)
	L. plantarum	Helped to increase body weight, cocoon, shell, and pupation rate	(Singh et al., 2005)
	Staphylococci		

	Staphylococcus gallinarum strain SWGB 7 & S. arlettae strain SWGB 16	Increased larval growth and cocoon characters (filament length and weight, finer denier)	(Saranya et al., 2019)
	Yeast		
	Saccharomyces cerevisiae	Immunomodulating agent; increased raw silk production with fewer cocoons; increased protein content	(Esaivani et al., 2014; Taha et al., 2017)20/10/2023 08:29:00
	Fungi		
	Trichoderma harzianumas	Improved food digestion leading to increased growth and resistance to mortality by *Metarhizium anisopliae* and *Beauveria bassiana*	(Alcosaba, 2019)
	Commercial products		
	Lact-Act [a]	Larvae reared on leaves sprayed with Lact-Act had increased survival when exposed to bacterial pathogens (*Bacillus thuringiensis* var. *sotto.* and *Staphylococcus aureus*)	(Rajakumari et al., 2007)
Insect species	**Probiotics**	**Effects on performance and yield**	**Ref.**
Greater wax moth	**Bacteria**		
Galleria mellonella	***Clostridiaceae***		
	Clostridium butyricum Miyairi *588*	Induced immune response and increased survival rates against *Salmonella enterica* serovar *Typhimurium,* enteropathogenic *Escherichia coli* or *Listeria monocytogenes.*	(Scalfaro et al., 2017)
	Lactobacilli		
	Lactobacillus acidophilus ATCC 4356	Increased survival from *Candida albicans* infection	(Vilela et al., 2015)
	L. kunkeei [b]	Reduces infection of *Pseudomonas aeruginosa* through biofilm formation and affecting their stability	(Berríos et al., 2018)

	L. rhamnosus ATCC 7469	Promoted greater protection in larvae infected with *Staphylococcus aureus* or *Escherichia coli*.	(Jorjão et al., 2018)
	L. rhamnosus ATCC 9595	Reduces infection of *Pseudomonas aeruginosa* through biofilm formation and affecting their stability	(Ribeiro et al., 2017)
	L. rhamnosus GG	Induced immune response and increased survival rates against *Salmonella enterica* serovar *Typhimurium,* enteropathogenic *Escherichia coli* or *Listeria monocytogenes.*	(Scalfaro et al., 2017)
Yellow mealworm	**Bacteria**		
Tenebrio molitor	***Bacilli***		
	Bacillus subtilis	Enhanced growth and nutritional fortification	(Rizou et al., 2022)
	B. toyonensis	Enhanced growth and increased dry matter weight of produced feed	(Rizou et al., 2022)
	Enterococcaceae		
	Enterococcus faecalis	Increased larval weight gain and overall size and shorter time to pupation, also increased the crude protein content	(Rizou et al., 2022)
	Lactobacilli		
	Pediococcus pentosaceus (Isolated from the gut of *Tenebrio* larvae)	Reduces mortality in larvae and accelerates the rate of development. The strain has antimicrobial activity towards a number of pathogenic bacteria including several *Bacillus thuringiensis, Serratia,* and *Pseudomonas spp*.	(Lecocq et al., 2021)
Insect species	**Probiotics**	**Effects on performance and yield**	**Ref.**
Black soldier fly	**Bacteria**		
Hermetia illucens	**Actinomycetia**		

	Arthrobacter AK19	Enhanced growth rate at early life stages culminating in larger larvae than control	(Kooienga et al., 2020)
	Bacilli		
	Bacillus subtilis S15 S16 S19; B. subtilis natto D1	Increased larval weight and total development time compared to control larvae	(Yu et al., 2011)
	Bifidobacteria		
	Bifidobacterium breve	Larvae had lower weights and appeared weak/slow/discolored compared to control	(Kooienga et al., 2020)
	Nocardiaceae		
	Rhodococcus rhodochrous	Increased conversion rate, which could result in larger larvae with less feed. Larvae had increased proteins content related to energy production and storage. Larvae without the probiotic which had higher content of proteins related to stress responses.	(Franks et al., 2021)
	Commercial product		
	Actisaf® Sc47 [c]	Increased bioconversion rate, lipid and protein yield in processed larvae	(Richard et al., 2019)
House fly	**Bacteria**		
Musca domestica	***Enterobacteriaceae***		
	Enterobacter hormaechei	Increased body length and weight, pupal weight, and shortened growth cycle, which is a considerable advantage that can contribute to cost savings and boost production in large-scale feeding facilities.	(Zhang et al., 2021)

[a]—Probiotic powder containing *Lactobacillus sporogens*, *Bacillus thuringiensis*, yeast hydrolysate, a-amylase, vita. min and mineral mix; [b]—Strain was isolated from honeybee guts and tested against gram—pathogen *Pseudomonas aeruginosa*; [c]—Yeast—*Saccharomyces cerevisiae* CNCM I-4407.

4.1. Isolating potential probiotic strains and their characterization

There is no standard method for identifying probiotic strains; however, potential probiotic candidates are usually identified in the core microbiota of the insect (Cai et al., 2018; Dierking and Pita, 2020; Yan and Polk, 2011), primarily through a metagenomic approach, as illustrated in Figure 1 The microbiota is readily characterized via DNA extraction followed by 16S rRNA

gene sequencing. The 16S rRNA gene region is used for sequencing, as it is a short (approximately 300–500 bases), conserved gene specific to bacterial genus, and even some species (Clarridge, 2004). However, as discussed in a recent review by Winand et al. (2019), next-generation sequencing such as Illumina and Nanopore Technologies offers a reliable identification of bacterial genera but can have reduced accuracy in the identification of bacterial species, necessitating the combination of omics with classical microbiological techniques to get down to species and strain level. Once the core microbiota is classified, further culturing steps can be utilized to target specific bacterial isolates. Yeruva et al. (Yeruva et al., 2020) utilized this approach when they assessed the midgut of *B. mori* to identify potential probiotics. Through this approach, *Enterococcus*, *Lactobacillus*, and *Bacillus* species were found to be dominant in the microbiota, and upon further evaluation, these species are well-known producers of coenzymes, antimicrobial substances and extracellular enzymes (Yeruva et al., 2020).

The analysis of health-promoting properties of probiotic bacteria and yeast should be conducted using in vitro and *in vivo* approaches before providing the strain on a large scale (World Health Organization and Food and Agriculture Organization of the United Nations, 2016). Papadimitriou et al. (2015) and Byakika et al. (2019) provided an overview on the assays that can be performed on bacterial strains for observing if the safety and biological and chemical characteristics of the strain fulfill the characteristics of being classified as probiotics. The same procedures have to be performed on yeast strains (Kumura et al., 2004).

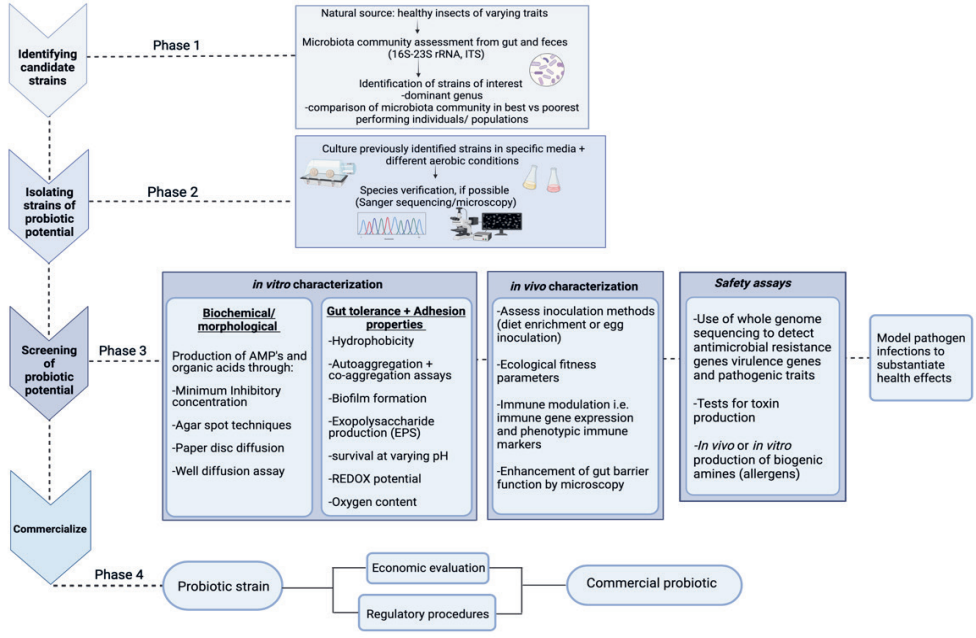

Figure 1. General workflow and screening techniques to characterize strains probiotic potential prior to commercialization. Phases 1 and 2 display the identification of candidate strains and isolation methods; phase 3 offers different *in vitro* and *in vivo* techniques for characterizing the probiotic potential. Fundamental *in vitro* safety assays are also listed. Phase 4 highlights two major factors that also need evaluation prior to the probiotic being used commercially.

4.1.1. Safety assays

All intrinsic characteristics relevant to the strains have to be fully evaluated before considering the strain safe for probiotic purposes. Safety assays on the production of biogenic amines by the decarboxylation of amino acids by substrate specific decarboxylases of potential probiotic bacteria should be conducted; for example, the production of histamine, which can persist in the food chain and lead to severe allergies through consumption of edible insects (Chomchai and Chomchai, 2018; Papadimitriou et al., 2015).

Moreover, the determination of the minimal inhibition concentrations (MIC) related to antimicrobials in probiotic strains reduces the risk of the addition of antimicrobial-resistant genes in the insects' gut environment and avoidance of the horizontal transmission of these genes to other microorganisms. The official protocols created by the European Food Safety Agency (European Food Safety Authority, 2012) have to be performed following international standard recognized methods cultivating the strain on seven antibiotics and the cut-off values, taking into considerations strain, growing conditions, and dilution variability (CLSI; www.clsi.org accessed on 2 February 2022; ISO; www.iso.org accessed on 15 January 2022). Other safety considerations include the production of virulent genes and toxin production, which could be deleterious to reared insects and consumers of insects.

4.1.2. Analysis of antimicrobial potential

The analysis of the antimicrobial potential is performed to assess the strain's ability to produce antimicrobial compounds (AMCs) active against selected pathogens and can be performed by running in vitro assays. The agar spot method is efficient for recording the probiotic's zone of pathogens' inhibition on selected media in selected growth conditions (Barbosa et al., 2005; European Food Safety Authority, 2012; Lecocq et al., 2021). The technique is used for quantifying the effect of antimicrobial agents such as bacteriocins and organic acids on selected pathogens. As agar gradient and culturing conditions can distort the effective concentration/production of the molecules, other methods can be applied. The paper-disk diffusion assay (Tagg and McGiven, 1971) and the well diffusion assay work on the same principle. For reducing the effect of the concentration of agar, liquid-medium techniques have been proposed by recording nisin production in *Leuconostoc mesenteroides* (De Moss et al.) (Cabo et al., 1999; Juturu and Wu, 2018). Microbiota interactions and environmental conditions such as pH and temperature can influence the production of antimicrobial compounds. For instance, in vitro studies highlighted optimal conditions of pH 6.2 and a temperature of 37 °C for some *Lactobacillus* spp. for producing bacteriocins (Yang et al., 2018). It is also important to take into consideration the consistent differences that are present within and between insect orders in terms of gut structure and gut environment, which also affect the production of AMCs (Engel and Moran, 2013).

As probiotics can decrease pathogen inference by competition, co-aggregation abilities can be recorded by in vitro assays based on absorbance measurements and fluorescence and radiolabelling detection (Collado et al., 2007). Other in vitro methods used for observing the microbiota interactions and competition for space acquisition are focused on autoaggregation and on the probiotic's effects on the pathogen's capacity to produce biofilms. The methods are based on spectroscopy measurements. The autoaggregation of *Lactobacillus* spp. varies from 10 to 23% influencing competition processes against pathogens, therefore decreasing the ability of microorganisms such as *L. monocytogenes* to produce biofilm and to infect the host (Byakika et al., 2019; (Hamden et al., 2020; Woo and Ahn, 2013). Hydrophobicity properties related to gut adhesion and colonization can be measured by performing spectrometry on isolated strains of the potential probiotic culture in several organic solvents (Byakika et al., 2019 ; Collado et al., 2008 ; Hamden et al., 2020). Another important parameter that can

influence gut colonization by adhesion is the production of exopolysaccharides (EPS). For in vitro analysis of the production of these molecules, an extraction followed by concentration estimation by phenol-sulfuric acid method is efficient (Hamden et al., 2020). The expression of these parameters increases the probiotic strain capabilities to compete with pathogens strains in gut space colonization by reducing the chance of incurring infection.

4.1.3. Assessing immune modulation

The influence of a potential probiotic strain on the immune response can be determined by monitoring the expression of important immune response genes, or by monitoring the expression of genes encoding immunologically important molecules by quantitative RT-qPCR (Yan and Polk, 2011). The innate immune system comprises a set of genes representing four immune system pathways (Toll, Imd, JNK and JAK/STAT) (De Gregorio et al., 2002). The most commonly investigated groups of genes for probiotic studies are antimicrobial peptides (AMP) and pattern-recognition receptors of the Toll and Imd pathways due to the receptors mediating host-microbiota communication (Dierking and Pita, 2020). Coupled with an assessment of immune-relevant gene expression, it is also common practice to collect hemolymph samples for measuring other immunomodulatory parameters. Ordinarily, these are assays involving measuring the level of phenoloxidase, total protein concentration, and total hemocyte counts and differential haemocytes circulating in the hemolymph after the administration of a probiotic (Franks et al., 2021; Tlak Gajger et al., 2020).

4.2. Ecological fitness assay

According to Peacock (2011) fitness can be most usefully understood as all aspects resulting in survival, not only the properties of reproductive success. Furthermore, Rosenberg and Bouchard (Rosenberg and Bouchard, 2005) clarify ecological fitness as interactions between organisms and environments. With this definition in mind, it is clear that the experimental design is critical when evaluating the effect of probiotics on overall fitness and health, as the results can be influenced by several factors: 1. method of delivery; 2. biological traits measured; 3. effect on different life stages; 4. pre-existing microbiota; and 5. diet (Deutscher et al., 2019). Additionally, due to the diversity of life-history strategies and environments in which insects inhabit, there can be species-specific influences within these factors, further establishing a criterion for assessing probiotics in insects complicated. Nevertheless, indicators of biological fitness are often measured by the overall longevity, mortality, fertility, and fecundity of the insect. For those insect species that are holometabolous, the weight of pupae and adult emergence rate can also be monitored, as can, in some cases, the flight capacity (Hamden et al., 2020).

Within these measurements, it can be important to distinguish the additive role of the probiotic on having an effect due to the interaction with the insect or simply as a source of nutrients. Some researchers have attempted to separate these responses by providing both dead and live probiotics. When assessing the effect of *Klebsiella oxytoca* and *Enterobacter* sp. AA26 as probiotics in larval and adult *C. capitata* mass-reared for SIT, Kyritsis et al. (2017) provided both dead (inactivated via heat treatment) and live bacteria. In doing this, they were able to differentiate the responses as an effect of the live bacteria, or not. A reduction in the developmental time of the immature stages was found for flies fed both dead and live *K. oxytica*-enriched diets, but a positive effect on flight ability was only demonstrated in individuals provided the live bacteria. Similarly, Gavriel et al., (2011) only found an increase in mating competitiveness in medfly adults provided live bacteria, with no beneficial effect on males fed dead bacteria. In general, this highlights the need to better understand the role of a supplement as either providing an additional source of nutrients, or as establishing in the gut and interacting with the host.

In summary, there is no 'best practice' for assessing the efficacy of probiotics in insects. However, assays involving the assessment of the effect of the probiotic on biological parameters have become a standard practice. An often-overlooked element in categorizing probiotic potential is the evaluation of the microbiota community composition after the administration of the probiotic as some strains are able to reset unbalanced microbiota phenotypes by modulating the host defense systems, as well as drive microbiome functional shifts. These shifts, if sufficient, could achieve a positive effect or trigger a decline in nutrients, energy, and metabolic activity of the insect and reduce overall growth and reproduction performance (Cai et al., 2018; Daisley et al., 2020).

5. Improving mass reared insect fitness by probiotic provision

The gut microbial composition has the potential to shape its host growth trajectory in a stressful environment. The manipulation of the gut microbial composition by providing selected probiotic feed additives in the rearing systems could be a way to improve insect fitness, reduce the effects of external factors such as stress, and reduce or altogether prevent the use of chemical growth promoters (Novick, 2021). Studies on aseptically reared insects have mainly focused on the role of specific strains on nutrient absorption in poor nutrient conditions. A study on the popular insect model *Drosophila melanogaster* highlighted the importance of the beneficial metabolic dialogue between *Lactobacillus plantarum* and *Acetobacter pomorum*. The provision of these bacteria determines the boosting of the host juvenile growth despite the malnutrition, by each providing essential metabolites such as lactate, essential amino acids, and anabolic metabolites that foster growth (Consuegra et al., 2020).

Even if the selection of specific strains for improving fitness performances is still ongoing for several mass-reared insect species, some results have already been obtained on *B. mori*'s body weight, cocoon, shell, and pupation rate with the addition of *Lactobacillus* species in the diet (Yeruva et al., 2020). *Saccharomyces cerevisiae*, *Staphylococcus gallinarum* and *Staphylococcus arlettae* provided on mulberry leaves resulted in better performance in *B. mori* (Saranya et al., 2019; Esaivani et al., 2014). Positive connections between the provision of the strain *Pediococcus pentosaceus* (Fig. 2) to *T. molitor* larvae and fitness performances have been proven allowing its definition as probiotic (Lecocq et al., 2021). The definition of a protocol for providing the strain on an industrial scale is ongoing. Initial studies on *T. molitor* have demonstrated that the provision of a mixed culture of probiotic bacteria can affect growth and weight gain positively (Zhong et al., 2017).

The primary interests of providing probiotics to *Hermetia illucens* are mainly focused on waste conversion and their positive effect on larval growth. *Arthrobacter* AK19 and *Rhodococcus rhodochrous* 21198 increased the protein digestion and absorption by 20–30% with no impact on the microbial community. On the other hand, the provision of *Bifidobacterium breve*, caused an increase of 50% of larval final weight, 20% lower waste conversion, and the suppression of microbial community diversity at a benchtop and industrial scale (Kooienga et al., 2020).

The nutritional content of the insects is affected by the manipulation of the microbial composition. The dry matter and crude protein percentage showed higher values in *T. molitor* larvae and *H. illucens*'s fatty acids compositions and presented a shift to polyunsaturated fatty acids (Kooienga et al., 2020; Zhong et al., 2017). The selection of targeted microorganisms plays a key role in shaping the microbial community and obtaining positive effects on fitness parameters.

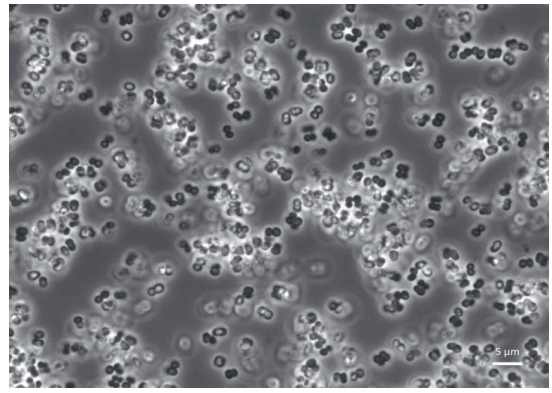

Figure 2. Confocal microscopy observation of *Pediococcus pentosaceus* KVBL 19-01. Probiotic activity of the strain has been recorded on *T. molitor* by Lecocq et al. (2021).

6. Concluding remarks and perspectives

The occurrence of diseases in mass-reared insects and the need of reducing the use of antibiotics in all the production systems to cope with the rising frequency of antibiotic resistance, are demanding new solutions for preserving animals' health and improving their fitness performances. The identification and the selection of host-specific probiotics could represent a sustainable solution for stabilizing insect production and ensuring food and feed safety.

Once the probiotic strain is selected, the supplements' formulation and the role of prebiotics in synbiotic interactions could have an important role in stabilizing the commercial product and in assuring the probiotic gut colonization and persistence after the provision to the insects.

Insect–microbiota relationships can affect behaviour and fitness performances in several stressful situations. Interest in the probiotic provision of insect species for food and feed purposes has already led to the enhancement of fitness performances and immune responses. Therefore, the chance to shape the microbial community favouring probiotic microorganisms by providing prebiotics in the diet increase the opportunities to promote the health status of the insects and to decrease the occurrence of diseases in mass reared conditions. A lot of questions related to synbiotic–host relationship are still open and multivariate statistical models are needed for studying the effects of diet, environmental factors, and microbiota on these interactions. Further studies could be focused on the manipulation of mass-reared insect microbiota for breeding individuals with a selected starting gut microbiota that could allow better growth and reproductive performance, decreasing the occurrence of diseases.

Author Contributions

C.S., L.M. and J.K.U. have equally participated to the conceptual design and the redaction of the manuscript.

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3

Chapter 3

Harmful and beneficial symbionts of *Tenebrio molitor* and their implications for disease management

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Abstract

The yellow mealworm, *Tenebrio molitor*, is currently one of the most important insect species produced for livestock feed and human consumption. High-density rearing conditions make the risk of disease and infections by parasitic symbionts a challenge in the mass production of these insects. However, certain symbionts are beneficial and should be favoured in order to promote healthy insect populations. Knowledge of parasitic symbionts and their management is essential for the insect rearing industry and its associated research. Here we review the documented microbial infectious agents, invertebrate parasites, and beneficial symbionts occurring in *T. molitor*. Furthermore, we discuss detection, prevention, and treatment methods for disease management in *T. molitor* production systems to inform future management and decision making in *T. molitor* rearing.

Keywords: mass-rearing, insect diseases, beneficial microorganisms, entomopathogens, probiotics

Introduction

The insect rearing industry has grown rapidly in recent years to meet the global demand for alternative and sustainable sources of feed and food (Francuski and Beukeboom, 2020). In 2017, 6,000 tons of insects were produced for animal feed in Europe alone (Derrien and Boccuni, 2018) and the global production of insects for food and feed is estimated to reach up to 500,000 tons by 2030 (de Jong, 2021). The yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), is one of the major insect species produced on a large scale due to its high protein and fat content, its efficient feed conversion rate and its comparatively simple rearing process (Costa *et al.*, 2020; Grau *et al.*, 2017b). Such characteristics make *T. molitor* an ideal candidate for addressing societal issues like sustainable food production and hunger. Consequently, the European Food Safety Authority (EFSA) (Reg EU 2021/882, Reg EU 2015/2083) permitted in 2021 the processing and commercialisation of dried *T. molitor* larvae for human consumption in Europe (European Commission, 2021).

One important challenge in the mass-production of insects is the risk of diseases and infections in these systems (Eilenberg *et al.*, 2018; Eilenberg *et al.*, 2015; van Huis, 2017). Mass-produced insects are generally kept at high densities. This favours the spread of microbial infectious agents and invertebrate parasites (either endo- or ectoparasites) between individuals if environmental conditions are conducive and no preventive measures are taken (Eilenberg *et al.*, 2018). Pathogens can be a major barrier in scaling up insect production, as well as an economic obstacle causing significant losses for insect production companies (Bhat *et al.*, 2009; Liu *et al.*, 2011). Furthermore, the ecological risk of pathogen spillover from mass-rearing facilities into natural insect populations further emphasises the importance of disease management (Bang and Courchamp, 2021).

Like in other animals, infectious diseases in insects are diverse and can be enigmatic, manifesting as lethal or sublethal, and as single or co-infections. Moreover, mutualistic and commensal symbionts can appear as etiological agents if their ecology is unknown, but in actuality are beneficial to the insect host, and in certain cases can protect the insects from disease (Lecocq *et al.*, 2021). Beneficial symbionts are therefore candidates for introduction as probiotics in mass-reared insects (Savio *et al.*, 2022).

To date, harmful and beneficial symbionts of *T. molitor* and their management have not been comprehensively reviewed. Understanding the variety of symbiotic interactions and their implications for disease outcomes is critical to the success of insect farming. Here we review the symbionts known to occur in *T. molitor*, and document the symptoms they cause, present detection methods, and discuss potential innovative treatment and prevention strategies.

Definitions of terms used in this review:

Symbiosis: Refers to any ecological relationship between two species, whether beneficial or detrimental to either partner, and is further subdivided into different forms (see below).

Mutualism: A symbiotic relationship in which both partners benefit from each other.

Commensalism: A symbiotic relationship in which one partner benefits and the other partner is neither harmed nor benefits.

Parasitism: A symbiotic relationship in which one partner causes harm to the other partner (Martin and Schwab, 2012).

Symbiont: An organism living in some form of symbiosis with another species.

Parasite: An organism living on or in a host organism, deriving nutrition at the expense of the host's health (Hajek and Shapiro-Ilan, 2018).

Pathogen: Microorganisms, including virus that causes, or can cause, disease (Pirofski and Casadevall, 2012).

Facultative pathogen or parasite: Pathogenic or parasitic symbionts capable of surviving and reproducing outside of a host organism.

Obligate pathogen or parasite: Pathogenic or parasitic symbionts reliant on a host organism for survival and reproduction.

Ecological host range: The sum of all the host species a parasite is capable of encountering and infecting in the natural environment; also refers to natural infection.

Physiological host range: The sum of all host species a parasite has been found to infect under laboratory conditions; also refers to experimental infection.

Harmful symbionts

In this section, parasitic symbionts harmful to *T. molitor* are addressed, although it is worth mentioning diseased states can be induced by a range of non-infectious factors, *e.g.* malnutrition (Kaya and Vega, 2012). Here, we distinguish between 'microbial infectious agents' (bacteria, fungi, protists, viruses) and 'invertebrate parasites' (mites, cestodes, nematodes, and parasitoids). Microbial infectious agents that infect insects are often also referred to as 'entomopathogens' or 'insect pathogens' in the literature (Eilenberg *et al.*, 2015; Hajek and Shapiro-Ilan, 2018).

Infections in insects manifest as acute or chronic and covert or overt. The most obvious and easily observable are acute infections, which are of short duration and may result in sudden death of the host. Chronic infections, on the other hand, are less apparent and can often be overlooked due to sublethal effects and the long incubation period before death. Covert or latent infections, primarily observed in viral infections, are a dormant form of disease, presenting no visible symptoms in the host. These have the potential to become overt or acute infections when triggered by appropriate abiotic or biotic factors, *e.g.* environmental effects (Hajek and Shapiro-Ilan, 2018) or transfer from one host to another (Martin and Brettell, 2019).

Parasites can occur in different life stages of *T. molitor* given the vast difference in body composition, behaviour, and environmental requirements throughout the insect lifecycle. For this reason, we specify the life stage in which each pathogen or parasite has been identified in *T. molitor* (Table S1). This classification should not be considered exhaustive, as bioassays may not have been performed on every life stage, rather it is the sum of current knowledge on *T. molitor* diseases.

Infections in insects can both be naturally occurring or experimentally induced. Given that the artificial environments in which *T. molitor* is mass-produced are ecologically novel relative to the evolutionary time scale on which symbiotic relationships have been formed, it is possible some parasites might be able to extend their ecological host ranges under these new conditions. We therefore include parasites that have *T. molitor* in their ecological as well as physiological host range to give a complete overview (Table S1).

Microbial infectious agents

Bacteria

The microbial communities of *T. molitor* are characterised by the presence of a resident microbiota, mainly composed of the bacterial phyla of Proteobacteria and Firmicutes, which can be shaped by feed and environmental conditions (Urbanek *et al.*, 2020). Bacteria can exploit different relationships with insects from mutualistic to pathogenic interactions (Vallet-Gely *et al.*, 2008). Assurance of safety for food and feed necessarily raises several questions regarding bacterial pathogens, including susceptibility, persistence, and transmission of pathogens in the host organism. This is especially relevant in mass-rearing systems characterised by high-density conditions and the practice of using organic side streams as insect feed (Jensen *et al.*, 2020; Maciel-Vergara *et al.*, 2021; Urbanek *et al.*, 2020; Wynants *et al.*, 2019). *Tenebrio molitor* is not naturally associated with food-borne and environmental bacterial pathogens considered infectious to humans, reducing their risk of acting as a biological vector between different trophic levels (Urbanek *et al.*, 2020).

Infection mainly occurs via oral ingestion of bacteria, although insect haemocoel can be directly infected when exposed through injuries or damage from fungi or nematodes (Maciel-Vergara *et al.*, 2018; Vallet-Gely *et al.*, 2008). A pathogenic bacterial species of *T. molitor, Bacillus thuringiensis* var. *tenebrionis,* was first isolated from *T. molitor* in 1982 (Krieg *et al.*, 1983) and is commercially used in biocontrol of certain pest species from Coleoptera. This gram-positive spore forming bacterium belonging to the phylum Bacillota is well known for causing death of the larval stages of many insects. Mortality occurs via septicaemia (Nielsen-Leroux *et al.*, 2012), when the insect gut is perforated through the action of bacterial pore forming toxins, following by infection of the whole body. Bacterial spores then germinate and proliferate throughout the haemocoel. In the case of *T. molitor* larvae, the extensive gut leakage has not been correlated to the killing mechanism of *B. thuringiensis,* but reduced feeding behaviour has been observed in infected individuals (Zanchi *et al.*, 2020). Other bacteria, such as *Serratia marcescens* (phylum Pseudomonadota), act mainly as opportunistic pathogens, when the insects are already physiologically weakened (Dupriez *et al.*, 2022).

A first indication of the presence of pathogenic bacteria is the observation of reduced feeding behaviour and decreased movement. In the case of bacterial proliferation and septicaemia, the insect cadavers change colour, presenting a flaccid consistency and a foul odour (Maciel-Vergara *et al.*, 2021). Specific insect colorations can sometimes be related to the presence of bacterial infection such as pink or red for *S. marcescens,* and dark colours for other bacterial species (Dupriez *et al.*, 2022; Eilenberg *et al.*, 2015). The application of molecular techniques such as Next Generation Sequencing (NGS) is recommended for identifying bacterial pathogens to the species level (Verma *et al.*, 2017).

Fungi

Several fungal species of the orders Hypocreales, Eurotiales, Capnodiales and Saccharomycetales have been shown to affect *T. molitor*. Most of the studies are based on experimental infections and focus on the application of entomopathogenic fungi as biocontrol agents for tenebrionid pest species. Fungal infections either lead to mortality of the insects or induce sublethal effects, such as a modification of the lipid composition (Gołębiowski *et al.*, 2020) or a negative effect on the number of offspring (Pedrini *et al.*, 2010) as shown in other species of Tenebrionidae. It is important to note that different fungal isolates from the same species can have highly variable virulence (Praprotnik *et al.*, 2021).

In insects, infections by fungi are typically transmitted when a spore from their surrounding environment encounters an appropriate insect host cuticle, and there is sufficient humidity and temperature for the spore to adhere and germinate (Vega *et al.*, 2012). The germinating spores penetrate the host cuticle by producing chitinase and induce mortality as fungal structures are produced throughout the host body (Vega *et al.*, 2012).

Within the order Hypocreales, members of the genera *Metarhizium* (Barnes and Siva-Jothy, 2000; Bharadwaj and Stafford, 2011; Keyser *et al.*, 2016; Keyser *et al.*, 2014; Korosi *et al.*, 2019; Mathulwe *et al.*, 2021; Moret and Siva-Jothy, 2003; Praprotnik *et al.*, 2021) and *Beauveria* (Korosi *et al.*, 2019; Lee *et al.*, 2014; Maistrou *et al.*, 2018) have been described to infect *T. molitor*. Many members of these two genera are generalist pathogens having many different host species (Maciel-Vergara *et al.*, 2021). They are highly relevant in production systems of *T. molitor* because they can be found in stored grains (Wakil *et al.*, 2014). Stored grains are not only a natural habitat of *T. molitor*, but they are also frequently used to feed *T. molitor* larvae in production facilities (Cortes Ortiz *et al.*, 2016). A typical symptom of *T. molitor* infected with fungi from the order Hypocreales is white fungal outgrowth when cadavers are kept at high humidity. Moreover, the fungi start to produce conidia after the fungal outgrowth (green conidia: *Metarhizium* spp.; white conidia: *Beauveria bassiana*). The genera can often be determined based on characteristics of the conidia using light microscopy (Maciel-Vergara *et al.*, 2021), whereas molecular methods are needed to identify the species (Castrillo and Humber, 2009).

Several fungal species not classified as entomopathogens have been shown to affect *T. molitor* when they were ingested together with the feed. Guo *et al.* (2014) found that *T. molitor* larvae fed with *Fusarium avenaceum*- and *F. culmorum*-colonised wheat kernels had an increased mortality compared to the control. This was despite having found none of the tested *Fusarium* species multiplied inside the insect haemocoel, indicating that mycotoxins produced by the fungi are responsible for the mortality of the larvae (Guo *et al.*, 2014). Other fungal species growing on grains (*Aspergillus niger*, *A. flavus*, *Penicillium expansum*, *Cladosporium herbarum*, *F. nivale*, *F. equiseti*, *F. roseum* and *F. tricinctum*) have been shown to inhibit the growth of *T. molitor* larvae (Davis *et al.*, 1975; Reiss, 1973). The growth inhibition by mycotoxins might be a combination of the effect of the toxins inside the insect and deterrence in feeding behaviour of the contaminated feed (Davis *et al.*, 1975).

Studies investigating the effect of the mycotoxin deoxynivalenol (DON) on *T. molitor* larvae report contradicting results. Jankovic-Tomanic *et al.* (2019) found growth inhibiting effects of larvae reared on wheat containing 4.9 to 25 mg/kg DON (Janković-Tomanić *et al.*, 2019). Other studies, however, describe no effect on weight gain when the larvae of *T. molitor* were fed with wheat flour containing up to 8 mg/kg (Van Broekhoven *et al.*, 2017) or even up to 12 mg/kg DON (Ochoa Sanabria *et al.*, 2019). This indicates that mycotoxins from different fungal species or strains might have different effects on the larvae of *T. molitor*. Additionally, the human pathogenic yeast species *Candida albicans* and *C. neoformans* have been found to cause mortality in *T. molitor* when directly injected into the haemocoel of the larvae (De Souza *et al.*, 2015).

Microsporidia

Microsporidia are intracellular obligate parasites considered to be most closely related to fungi based on recent phylogenetic studies (Capella-Gutiérrez *et al.*, 2012) and are common parasites of insects: 93 of the 200 described genera of microsporidia have an insect as a host (Becnel and Andreadis, 2014). The most common pathway of microsporidia transmission to a new insect

host is through direct oral ingestion of infectious spores, which are found in food, faeces, or liquids within the host's immediate environment (e.g. soil, water, plant, insect cadaver). Vertical transmission, where infection is transferred directly from parent to progeny, can also occur in the case of transovarial transmission (Becnel and Andreadis, 2014).

Microsporidian infection in *T. molitor* thus far appears to be rare, with the only account of natural infection reported by Armitage and Siva-Jothy (2005), who identified unnamed microsporidians in a *T. molitor* lineage. 87% of the beetles in the infected culture carried microsporidia, although they determined the infections were not harmful for the insect (Armitage and Siva-Jothy, 2005). Fisher and Sanborn (1962) experimentally induced infection in *T. molitor* using the microsporidium *Paranosema whitei* (natural host: *Tribolium* spp.) with infected feed, but susceptibility to infection was limited to second and third-instar larvae immediately post-moult (Fisher and Sanborn, 1962). Moreover, Milner also found that first instar *T. molitor* larvae were not susceptible to *P. whitei* infection (Milner, 1973). Based on the limited evidence of the ability of microsporidians to induce diseased states in *T. molitor,* further research is required to evaluate the disease-risk of microsporidians in mass-rearing systems.

Protists

Protists, historically called protozoans, are an informal group describing free-living or parasitic single-celled eukaryotes other than fungi, animals, and plants. They are found in a myriad of cellular forms with diverse biochemistries, which allow them to colonise every biome and many different types of hosts. Multiple protistan groups have the capacity to infect animals and may cause serious disease (Lange and Lord, 2012). However, compared to prokaryotic microbes, fungi, and viruses, protists are often overlooked as potential pathogens of mass-reared reared insects (Bessette and Williams, 2022; Garofalo *et al.*, 2019; Maciel-Vergara *et al.*, 2021).

In insects, protists parasites typically start their life cycle when their environmental cysts or spores are ingested by a susceptible host (Lange and Lord, 2012). All protists identified as symbionts of *T. molitor* are typically transmitted via this route, causing infection after oral ingestion of the infectious stages of the protist. Infection by protistans is not typically obvious, as it is generally chronic with no external indications of disease. An infection of high intensity with neogregarines or coccidians can cause insects to become lethargic with a swollen, whitish appearance (Lange and Lord, 2012). The detection and identification of protist parasites has historically relied on microscopy and morphological identification in combination with knowledge of biological parameters, such as host specificity, tissues tropism, and route of infection (Solter *et al.*, 2012). Presently, the use of polymerase chain reaction (PCR) and, more recently, NGS, are being widely applied for discovering novel protist lineages and to understand their contribution to microbiomes (Bass and del Campo, 2020). The 18S (small subunit) ribosomal RNA gene (18S) is the most extensively used genetic barcode for protist surveys (Vaulot *et al.*, 2022).

Amoebozoa

The Amoebozoa group includes unicellular eukaryotes that possess pseudopodia for their motility and ingestion (Anderson, 2017). Six amoebae species are reported to be parasitic to insects. Moreover, it is possible for insects to act as mechanical vectors for amoebae pathogenic to humans and other animal hosts (Lange and Lord, 2012). Entomopathogenic amoebae are known to form resilient uninucleate cysts in the environment, that will excyst within a suitable host and release trophozoites (i.e., active feeding stages) found either in the midgut or

Malpighian tubules (Lange and Lord, 2012). New formed cysts are then released in the environment through the insect frass. Specific parasitic amoebae of the mealworm have yet not been described, but *T. molitor* has been found to carry *Entamoeba* spp., with the amoebae species *E. histolytica* known to cause dysentery to humans (Gałęcki and Sokół, 2019) (Table S1).

Coccidia

Coccidians, also called haemogregarines, are similar to neogregarines. These endoparasites primarily infect vertebrates but are also found in invertebrates, with less than 1% of the described species infecting insect hosts (Lange and Lord, 2012). Few studies have examined coccidians within *T. molitor*, but some species are known to infect other Tenebrionidae, such as *Adelina castana* and *A. picei,* parasites of *Tribolium castaneum* and *Alphitobius piceus*, respectively (Ghosh *et al.*, 2000). *Adelina* spp. are not well studied in *T. molitor*, and only one reference from 1930 has reported a natural infection from reared *T. molitor* with *Adelina tenebriosis* (Sautet, 1930). The potential effects of coccidian infections on *T. molitor* are not studied, but lag in development time has been reported in another Tenebrionidae, which could impact insect production (Park T, Frank MB. The Population History of Tribolium Free of Sporozoan Infection. The Journal of Animal Ecology. 1950 Nov;19(2):95.)

Cryptosporidia

Recently, *Cryptosporidium* has been proposed as a gregarine (Adl *et al.*, 2019) but later this was abandoned to place *Cryptosporidium* as a basal group of apicomplexans (Salomaki *et al.*, 2021). Interestingly, *Cryptosporidium* spp., a vertebrate parasite, can be found on the surface or in the intestines of different insects, which could serve as mechanical vectors. A parasitological evaluation undertaken in European farms (with insect stock from all over the world) has reported that *T. molitor* could be a vector of this pathogen, presenting a risk for human health (Gałęcki and Sokół, 2019). The same study also found *Isospora* spp. in *T. molitor*, a coccidian pathogen that can induce gastrointestinal symptoms in humans, livestock, and exotic animals (Gałęcki and Sokół, 2019).

Gregarines

The most well studied protist group infecting *T. molitor* is Gregarinasina, known commonly as gregarines. Gregarines are the most abundant group of Apicomplexa that infect invertebrates (Desportes and Schrével, 2013; Votýpka *et al.*, 2017). Gregarines are mainly extracellular parasites and attach to the host via anchoring structures which allow them to feed on host cell cytoplasm (Lange and Lord, 2012). Two main orders compose the subclass Gregarinasina, the Eugregarinorida and the Neogregarinorida. In contrast to the eugregarines, the neogregarines (syn. schizogregarines) can develop intracellularly in the host tissues. This development induces more serious disease than eugregarines, as the intensity of infection of the eugregarines is limited to the number of oocysts that are ingested by the host (Lange and Lord, 2012).

Five species are known to infect *T. molitor*: *Gregarina polymorpha*, *G. niphandrodes*, *G. cuneata*, *G. steini* and *Mattesia* spp. (Berndt, 1902; Clopton *et al.*, 1991; Hammerschmidt, 1838; Harry, 1967; Kleespies *et al.*, 2008; Koura and Kamel, 1993; Lipa, 1967; Rodriguez *et al.*, 2007; Schawang and Janovy, 2001; Stein, 1848; Valigurova, 2012). Gregarines are naturally present in Tenebrio population with controversial effect on their host. Rueckert *et al.*, 2019 did an extensive review on the effects of gregarines and how they transgress the symbiosis spectrum. Two references showed positive effects on *T. molitor* infected by *Gregarina sp.* with

enlarged host growth and positive impact on host development, fitness and longevity (Sumner, 1936; Valigurova, 2012). Other studies showed negative or no effect on the development and fitness of *Tenebrio* hosts.

Viruses

The first report of a virus naturally infecting *T. molitor* dates back to 1969 (Huger, 1969). Viral particles similar to densovirus were identified in diseased larvae using an electron microscope, however, no further molecular analysis was carried out (Huger, 1969). These larvae presented a grey discoloration and cytopathic modifications on diverse tissues such as the epidermis, or the fat body. Moreover, *T. molitor* may act as a mechanical vector for Acheta domesticus densovirus (AdDV). AdDV-positive *T. molitor* individuals were detected in a colony reared together with infected house crickets (*Acheta domesticus*) within the same facility (Szelei *et al.*, 2011). These results indicate the possibility of horizontal transmission of densovirus between insect species. Apart from densovirus, viruses of the family Iridoviridae were found and demonstrated to be capable of infecting *T. molitor*. Particles of small iridescent virus (type 29) were identified in *T. molitor* larvae using electron microscopy (Black *et al.*, 1981; Kelly *et al.*, 1979). Wild type and recombinant invertebrate iridescent virus 6 (IIV6) have also been shown to cause disease in *T. molitor* via injection. Symptoms of infection include paralysis of larvae and darkening of cadaver three days after infection (Gencer *et al.*, 2020).

Regarding detection, molecular techniques are the most suitable method to correlate viral infection with the disease symptoms. Moreover, the advent of high-throughput sequencing has spurred the discovery of covert viruses in insects (Käfer *et al.*, 2019; Shi *et al.*, 2016; Wu *et al.*, 2020), including mass-reared edible species (Bertola and Mutinelli, 2021). Most of these viruses infect the host in a covert state with no visible biological costs. Therefore, it is likely that the number of viruses described for *T. molitor* and related species increases in the near future. In this scenario, analysing the risk of a potential viral outbreak and the sublethal effects caused by covert infections will be of high value to assess the level of risk for the mass-rearing industry.

Invertebrate parasites

Acari

Acari are ectoparasites capable of colonising many orders of insects. A parasitological evaluation of farmed insects found that *T. molitor* can carry mites belonging to the Acaridae (Gałęcki and Sokół, 2019). However, further research is needed to determine whether mites are parasites of *T. molitor*. Furthermore, mite debris should be considered as a potential hazard in insects produced for human consumption due to dust mite allergies. Other Tenebrionidae, such as *Alphitobius diaperinus* are known to be parasitised by *Acarophenax mahunkai*, which feed on the eggs of this species (Steinkraus and Cross, 1993).

Cestodes

Cestodes are a group of intestinal endoparasites. In their adult stage, cestodes mainly affect vertebrates. However, the larval stages (cysticercoids) can infect invertebrates via oral ingestion as intermediate hosts while they develop their infective capacity on the definitive host (Saari *et al.*, 2019). Most literature concerning cestodes focuses on the family Hymenolepididae. Natural infections of this cestode have been reported in *T. molitor* and other species of Tenebrionidae such as *Tribolium castaneum* and *Tr. confusum* (Heyneman and

Voge, 1971; Hurd *et al.*, 1990; Makki *et al.*, 2017) but most of the studies report experimental infections.

Although cestodes are not considered direct insect parasites, some studies have shown sublethal effects of cestode infection in the insect host. For instance, infection with *Hymenolepis diminuta* reduced the locomotion of *T. molitor* larvae in comparison to healthy individuals (Hurd and Parry, 1991; Hurd *et al.*, 1990; Sheiman *et al.*, 2006). Moreover, infection with *H. diminuta* was related to a decrease in the reproductive vigour of infected males and the fertility of females (Cole *et al.*, 2003; Hurd and Parry, 1991; Maema, 1986). In addition, we should avoid the presence of cestodes in mass-reared insects for assuring the food safety of the final product (Boelaert *et al.*, 2021).

To detect the infective stage of cestodes in insects, the use of a light microscope is recommended, while the application of molecular techniques may be required for characterisation at the species level.

Nematodes

No natural nematode infections have been described in *T. molitor* to date. However, diverse studies have assessed the physiological host range and pathogenicity of nematodes through experimental infection. These studies, which aim to unravel the potential of nematodes as biocontrol agents, concluded that several nematode species can infect *T. molitor* when added to the diet (de Carvalho Barbosa Negrisoli *et al.*, 2013; Ramos-Rodríguez *et al.*, 2006; Shapiro-Ilan *et al.*, 2008) (Table S1). Species belonging to the genera *Steinernema* and *Heterorhabditis* are the main entomopathogenic nematodes described in *T. molitor*. These nematodes require high moisture conditions for infection (Eilenberg *et al.*, 2015) and desiccation tolerance is strain dependent (Shapiro-Ilan *et al.*, 2014). Similarly, the heat tolerance and the virulence of nematodes are influenced by the behavioural and physiological characteristics of the specific isolates and the environmental conditions (Lulamba and Serepa-Dlamini, 2020; Ramakuwela *et al.*, 2018). Members of the *Oscheius* spp. are also capable of infecting *T. molitor* both through experimental infection and using *T. molitor* as a bait (Foelkel *et al.*, 2017; Torrini *et al.*, 2015).

Nematode infections can be directly detected using a magnifying lens, while the species characterisation requires the application of molecular techniques.

Parasitoids

Like nematodes, no natural parasitoid infection of *T. molitor* has been described to date. However, due to its high accessibility and low production costs, *T. molitor* has been used as an alternate factitious host to rear multiple parasitoid species for biocontrol. Several studies were conducted to investigate the use of *T. molitor* as a host for rearing parasitoids at various life stages, including different pupal ages and eggs (Table S1). *Tenebrio molitor* was demonstrated to be a highly suitable host for rearing parasitoids of lepidopteran species, with the level of parasitism in *T. molitor* reaching 100% of efficacy and an emergence rate above 90% (Andrade *et al.*, 2012; Favero *et al.*, 2014; Zanuncio *et al.*, 2008).

Beneficial symbionts

Mutualistic associations between hosts and their microbiota are well-known in the animal kingdom. Several microorganisms, especially prokaryotes, have been shown to have beneficial effects on *T. molitor,* all of which increased the growth of the larvae (Table 1). Reiss et al

(1973) demonstrated other positive effects such as increased larval survival and adult emergence was conferred by *Pediococcus pentosaceus* when provided in both vital and inactivated form (Lecocq *et al.*, 2021). *Bacillus subtilis*, *Bacillus toyonensis*, and *Enterococcus faecalis* had effects on the nutritional contents of the larvae (Rizou *et al.*, 2022), all of which increased crude protein content. Additionally, it has been shown that the gut biome of *T. molitor* larvae affects the parasite establishment of the tapeworm *Hymenolepis diminuta* (Fredensborg *et al.*, 2020). Controversial effects have been recorded on Gregarines impact on host development, fitness and longevity. Sumner (1933, 1936) and Valigurova (2012) observed an increased larval growth and longevity in *T. molitor* larvae infected with Gregarina *spp.*

Bacteria also present the possibility for use as probiotics to prevent diseases in reared insects, as is practiced in other livestock populations (Grau *et al.*, 2017b; Savio *et al.*, 2022). Probiotics are usually bacteria that either inhibit parasites (*e.g.* via inhibition of the expression of virulence genes or the increased production of antimicrobial substances) or increase the resistance of the insects by the stimulation of the host immune response (Grau *et al.*, 2017b). The *in vivo* application of probiotics to make *T. molitor* more resistant to parasites has not been demonstrated thus far. However, in the red flour beetle *Tr. castaneum*, the feeding of a probiotic (*Enterococcus mundtii*) increased the survival of larvae after exposure to *B. thuringiensis* (Grau *et al.*, 2017a), and *in vitro* studies of *P. pentosaceus* demonstrated growth-inhibiting effects on different entomopathogenic bacteria (*Bacillus thuringiensis*, *Serratia marcescens*, *Serratia plymuthica* and *Pseudomonas aeruginosa*) (Lecocq *et al.*, 2021).

Table 1. Overview of beneficial symbiont species and their effects on *T. molitor*.

Classification	**Species**	**Effect on *T. molitor***	**References**
Bacteria	*Enterococcus faecalis*	Increased larval growth, reduced larval development time, increased crude protein content of larvae	(Rizou *et al.*, 2022)
	Bacillus subtilis	Increased larval growth, increased crude protein content of larvae, decreased crude fat content of larvae, decreased microbial counts of *Enterobacteriaceae*	(Rizou *et al.*, 2022)
	Bacillus toyonensis	Increased larval growth, increased crude protein content of larvae, decreased microbial counts of *Enterobacteriaceae*	(Rizou *et al.*, 2022)
	Pediococcus pentosaceus	Increased larval survival and growth, increased adult emergence	(Lecocq *et al.*, 2021)
	Mixed culture of *Bifidobacterium bifidum*, *Clostridium butyricum*, *Bacillus subtilis* and *Bacillus licheniformis*	Increased larval survival and growth, increased crude protein content of larvae, decreased calcium and phosphorus contents of larvae	(Zhong *et al.*, 2017)
Fungi	*Neurospora sitophila*	Increased larval growth	(Reiss, 1973)
	Pithomyces chartarum	Increased larval growth	(Davis *et al.*, 1975)
Gregarinasina	*Gregarina* spp.	Increased larval growth and longevity	(Sumner, 1933; 1936; Valigurova, 2012)

Implications for mass-rearing systems

Methods for detection and isolation

Diagnostic techniques used in detection, identification, and characterisation of parasites in diseased insects have evolved considerably over the past decades. Conventional methods to examine these parasites include microscopic analysis, observation of the respective signs and symptoms, and isolation using specific selective media (Bing *et al.*, 2021; Vandeweyer *et al.*, 2021). Gałęcki and Sokół (2019) demonstrated the use of microscopic analysis and Ziehl-Neelsen application of staining methods (Carter and Cole Jr, 2012) in identifying various parasites in *T. molitor* production facilities (Gałęcki and Sokół, 2019).

However, many of these parasites are unculturable (Masson and Lemaitre, 2020) on artificial media (e.g. *Ichthyosporea* spp.). In addition to that, parasites like protists and viruses may be present in covert states, presenting no observable signs or symptoms. The advent of new technologies in the field of molecular biology allows for the identification of these parasites via PCR by targeting parasite-specific genome regions. For example, by amplifying the non-structural protein 1 (NS1) coding region in densovirus, it was possible to identify AdDV positive *T. molitor* colonies reared together with *A. domesticus* within the same facility (Szelei *et al.*, 2011). In another case, confirmation of the presence of IIV6 in *T. molitor* larvae was determined using PCR in larvae showing symptoms of paralysis (Gencer *et al.*, 2020).

The rapid development of NGS technology in recent years allows for the detection of unsuspected and novel parasites via a metagenomics approach, as well as providing the possibility to simultaneously assess the microbiome and macrobiome of species of interest (Gibson *et al.*, 2014). A general estimate of the relative abundance of particular organisms within a sample is also possible using metagenomic approaches, which can help determine the clinical significance of a parasite of interest. The initial culturing procedure or preliminary knowledge of signs and symptoms of parasites are not necessary in this technique (Frey and Bishop-Lilly, 2015). The use of metagenomics has already revealed novel pathogens in several commonly reared insect species, such as the presence of a new iflavirus in *A. domesticus* colonies (de Miranda *et al.*, 2021). Reference databases of parasite genomic sequences are crucial for untargeted metagenomic screening approaches (de Miranda *et al.*, 2021). Regardless of the detection method, storage conditions such as temperature (-80°C) prior to analysis are essential to maintain the stability of the genetic material for long periods (Bing *et al.*, 2021; Yang *et al.*, 2021).

In many cases, conventional detection methods are sufficient to identify common parasites with well-described signs and symptoms. Routine surveillance can be performed for the detection of previously recorded parasites in insect farming with standard PCR assays. In scenarios where unknown or suspected chronic diseases covertly reduce the fitness of the insects, the metagenomics approach is helpful in discovering the potential causative agent. In the future, it is possible that NGS techniques could be used to detect diseases even before infection, for example in the feed or in the circulating air (Sikorowski and Lawrence, 1994; Szelei *et al.*, 2011).

Management of harmful symbionts

Previous reviews and protocols of measures and good practice used in insect mass-rearing systems provide a useful framework for prevention and management of diseases in insects (Eilenberg *et al.*, 2015; International Platform of Insects for Food and Feed, 2022; Maciel-

Vergara *et al.*, 2021; Maciel-Vergara and Ros, 2017). These general guidelines include hygiene and facility design, and are largely applicable to the production of *Tenebrio molitor.* While diligent hygienic practices are the most important aspect of disease prevention in insect production, research into new prevention methods is continuously ongoing. Here we focus on research with future potential for innovative methods in the context of *T. molitor* disease management.

Insights in insect ecology offer promising potential for managing disease in the future. For example, it has recently been discovered that insects have a form of innate immune memory called 'immune priming', which protects them from pathogens when previously exposed to a pathogen or a pathogen-derived compound (Little and Kraaijeveld, 2004; Vigneron *et al.*, 2019). Several authors have suggested making use of immune priming in the commercial production of insects (Grau *et al.*, 2017b; Maciel-Vergara and Ros, 2017). The application of immune priming has been shown to be successful in another invertebrate system, the production of giant tiger prawns (*Penaeus monodon*), providing protection from infections caused by white spot syndrome virus (Witteveldt *et al.*, 2004). In *T. molitor,* immune priming has been shown to have both intra- and transgenerational effects (Dhinaut *et al.*, 2018). Immune priming of *T. molitor* using gram-positive bacteria conferred protection from infections with pathogenic gram-positive and –negative bacteria within generation and the next generation (Dhinaut *et al.*, 2018). To reduce the risk of insects becoming infected during the immune priming treatment, heat inactivated microorganisms could be used, as it has been successfully demonstrated in *T. molitor* larvae (González-Acosta *et al.*, 2022). This could be useful in the future as a preventative treatment for parasites known to be problematic in insect facilities.

Modification of diet components might be another useful tool to prevent or treat parasites. For example, beneficial compounds found in diets, like flavonoids, could confer protection from parasites. In a study on amoeba in locusts, hosts collected in the field had lower infection rates by the amoeba *Malamoeba locustae* (Abdel Rahman *et al.*, 2015), compared to reared hosts (King and Taylor, 1936; Kleespies *et al.*, 2010). Abdel Rahman *et al.,* (2015) hypothesised that orthopterans living in natural conditions acquired immunity associated with feeding on the plant *Portulaca oleracea*, which contains flavonoids with potential anti-protist properties. Moreover, prevention of harmful symbionts could also be achieved by providing probiotics, as discussed in section 3.

Temperature treatments could prevent and treat disease outbreaks in *T. molitor* populations, in particular heat shock. Curative heat treatments can, for example, reduce the effects of viral pathogens in insects (Cevallos and Sarnow, 2010; Inoue and Tanada, 1977). Another interesting finding in this regard is that temperature stress can pre-emptively increase the immune responses of insect hosts and thereby decrease the susceptibility to pathogen infection (Browne *et al.*, 2014; Wojda and Taszłow, 2013). These findings have, however, not been tested in *T. molitor* thus far.

Previous work in selective breeding has shown it is possible to fix particular traits and produce lines of *T. molitor* with altered phenotypes (Song *et al.*, 2022). In the future, selective breeding of *Tenebrio* for the purposes of withstanding certain conditions or diseases might be of interest to insect producers. The knowledge necessary for producing resistant lines of insects will be developed from our understanding of different aspects that contribute to disease resistance, like insect behaviour, ecology, and evolution. For example, it might be possible to promote grooming behaviours, which is important in high-density conditions, based on our understanding of insect grooming.

Concluding remarks

The mass-production of *T. molitor* is a relatively young industry, and information on symbionts of this insect species is therefore still limited. However, *T. molitor* has been used as a model organism to study host-parasite interactions for several decades, providing valuable insights into its life history and ecology (Barnes and Siva-Jothy, 2000; Dhinaut *et al.*, 2018). In the future, it will be important to consider how diseases are classified in terms of host range, as insect rearing facilities are neither natural conditions nor optimised laboratory conditions. Under unnatural, high-density breeding conditions, it is possible that new and emerging parasites may adapt to infect insects that were formerly only capable of colonisation under experimental circumstances. It is largely unknown what effects altered environments like mass rearing facilities will have on host-parasite interactions and disease outcomes, which could be positive or negative for insect production. For this reason, it is important to understand a parasite's physiological as well as ecological host range, and the environmental and evolutionary forces driving adaptation and host-shifts. Interactive effects arising from co-infection must also be considered. Different parasite species or strains might infect simultaneously, resulting in unpredictable outcomes that are impossible to determine when studying parasites individually (Cory and Deschodt, 2018).

As the mass production of *T. molitor* grows alongside global demand for insect protein, it will be important to maintain awareness of the type and severity of organisms affecting insect stocks. This is especially true of disease-causing agents, given that mass-rearing is practised at high insect densities that are conducive to outbreaks. Likewise, continued research into the possible benefits of mutualistic organisms will also help to ensure the health and well-being of farmed insects. These areas of research could largely benefit from partnerships between academic institutions, government programs, and industry in order to identify and address emerging parasites of particular concern and ensure the best practices for maintaining insect health are known and implemented.

Author contributions

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Supplementary material

Table S1. Overview of *T. molitor* parasites, symptoms, detection methods, and prevention or treatment. Prevention and treatment are only mentioned if they are specific to the parasite, general prevention strategies are stated in the text.[a]

Classification	Genus *Species* and affected life-stage (E, L, P, A)[b]	Symptoms	Detection method	Prevention and/or treatment	References
Acaridae	n.i.a.	n.i.a.	Microscopic examination.	n.i.a.	(Gałęcki and Sokół, 2019)
Amoebozoa	***Entamoeba*** spp. (A) including *E. coli*, *E. dispar*, *E. hartmanii* and *E. histolytica*	n.i.a.	Microscopic examination of homogenised gut and remaining tissues.	Maintenance of high hygienic levels in the production site prevents the transmission of these parasites from humans or animals.	(Gałęcki and Sokół, 2019)
Bacteria	***Aeromonas*** *A. hydrophila*[1] (L)	n.i.a.	n.i.a.	n.i.a.	1. (Noonin *et al.*, 2010) 2. (Dhinaut *et al.*, 2018) 3. (Krieg *et al.*, 1983) 4. (Wu and Dean, 1996) 5. (Zanchi *et al.*, 2020) 6. (Castro-Vargas *et al.*, 2017) 7. (Dupriez *et al.*, 2022)
	Bacillus *B. thuringiensis*[2,3,4,5] (L, A)	Decreased movement, cadavers change colour, become flaccid and malodourous	Microscopic examination of haemocoel of cadavers (the vegetative cells are typically rod like bacilli. Toxin crystal(s) and spores can be found in early infections or at a later stage).		
	Micrococcus *M. lysodeikticus*[6] (A)	n.i.a.	n.i.a.		
	Serratia *S. entomophila*[2] (A) *S. marcescens*[7] (L)	n.i.a. pink or red coloration of cadavers	n.i.a.		
Cestoda	***Hymenolepis*** *H. diminuta* (A)	Decreased fecundity, decreased activity and photophobic behaviour	Microscopic examination of homogenised gut and remaining tissues.	n.i.a.	(Cole *et al.*, 2003; Fredensborg *et al.*, 2020; Gałęcki and Sokół, 2019; Hurd and Fogo, 1991; Hurd and Parry, 1991; Hurd *et al.*, 1990; Sheiman *et al.*, 2006)

Ciliophora	***Balantidium*** spp. (A)	n.i.a.	Microscopic examination of homogenised gut and remaining tissues.	Keeping pest insects out of the rearing facility prevents transmission through insect vectors.	(Gałęcki and Sokół, 2019)
Cryptosporidia	***Cryptosporidium*** spp. (A)	n.i.a.	Microscopic examination of homogenised gut and remaining tissues.	Insects can serve as mechanical vectors for these vertebrate pathogens. Maintenance of high hygienic levels in the production site prevents transmission of these parasites.	(Gałęcki and Sokół, 2019)
Coccidia	***Adelina*** *A. tenebrionis*[1] (L, A) ***Isospora*** spp.[2] (A)	n.i.a.	Microscopic examination of homogenised gut and remaining tissues.	Insects can serve as mechanical vectors for these vertebrate pathogens. Maintenance of high hygienic levels in the production site prevents the transmission of these parasites.	1. (Sautet, 1930) 2. (Gałęcki and Sokół, 2019)
Fungi	***Aspergillus*** *A. flavus*[1] (L) *A. niger*[1] (L)	Growth inhibition	First diagnosis can be done by observing conidia or hyphae on cadavers.	The germination of conidia of most fungi is only possible under high relative humidity conditions - lowering relative humidity decreases the risk of infection. Different fungicides (the most effective was fluazinam) have been tested to treat *B. bassiana* in *T. molitor* larvae by adding the fungicides to the diet. Alternatively, fungicides could be used to disinfect the rearing environment (parts that larvae do not come in contact with).	1. (Reiss, 1973) 2. (Korosi *et al.*, 2019) 3. (Lee *et al.*, 2014) 4. (Maistrou *et al.*, 2018) 5. (De Souza *et al.*, 2015) 6. (Guo *et al.*, 2014) 7. (Davis *et al.*, 1975) 8. (Barnes and Siva-Jothy, 2000) 9. (Moret and Siva-Jothy, 2003) 10. (Bharadwaj and Stafford, 2011) 11. (Praprotnik *et al.*, 2021) 12. (Keyser *et al.*, 2014) 13. (Keyser *et al.*, 2016) 14. (Mathulwe *et al.*, 2021)
	Beauveria *B. australis*[2] (L) *B. bassiana*[2,3,4] (L, A) *B. pseudo-bassiana*[2] (L)	White fungal outgrowth and subsequent production of white conidia			
	Candida *C. albicans*[5] (L) *C. neoformans*[5] (L)	n.i.a.			
	Cladosporium *C. herbarum*[1] (L)	Growth inhibition			
	Fusarium *F. avenaceum*[6] (L) *F. culmorum*[6] (L) *F. equiseti*[7] (L) *F. nivale*[7] (L) *F. roseum*[7] (L) *F. tricinctum*[7] (L)	Growth inhibition			
	Metarhizium *M. anisopliae*[8,9] (L,A) *M. brunneum*[2,10,11,12] (L) *M. flavoviride*[2,13] (L) *M. guizhouense*[2,11] (L) *M. majus*[14] (L) *M. pingshaense*[2] (L) *M. robertsii*[2,11,12] (L)	White fungal outgrowth and subsequent production of green conidia			

	Penicillium *P. expansum*[1] (L)	Growth inhibition			
Ichthyosporea	n.i.a. (A)	n.i.a.	Microscopic examination of fat body, testes, and ventral nerve chord.	n.i.a.	(Lord *et al.*, 2012)
Insecta	***Chouioia*** *C. cunea*[1] (P) ***Palmistichus*** *P. elaeisis*[2,3] (P) ***Scleroderma*** *S. guani*[4] (P) *S. sichuanensis*[5] (P) ***Tetrastichus*** *T. howardi*[6] (P) ***Trichogramma*** *T. evanescens*[7] (E) ***Trichospilus*** *T. diatraeae*[8] (P)	n.i.a.	n.i.a.	n.i.a.	1. (Li *et al.*, 2019) 2. (Pereira Costa *et al.*, 2020) 3. (Zanuncio *et al.*, 2008) 4. (He *et al.*, 2006) 5. (Zhuo *et al.*, 2016) 6. (Tiago *et al.*, 2019) 7. (Salt, 1938) 8. (Favero *et al.*, 2014)
Microsporidia	***Paranosema*** *P. whitei* (L)	n.i.a	Microscopic examination of spores in gut tissues at x400 magnification	n.i.a.	(Fisher and Sanborn, 1962)
Gregarinasina	***Gregarina*** *G. cuneata*[1,2,3,4] (L, A) *G. niphandrodes*[5,6,7] (A) *G. polymorpha*[2,4,8,9] (L) *G. steini*[10] (L)	Damaged gut epithelial cells Lowered longevity of highly infected hosts n.i.a. n.i.a.	Microscopic examination of gut material and faeces.	Antibiotic treatment has been suggested for eliminating the presence of gregarines. *Gregarina* spp. have also been described as beneficial to the host by certain authors (see table 2)	1. (Lipa, 1967) 2. (Stein, 1848) 3. (Valigurova, 2012) 4. (Koura and Kamel, 1993) 5. (Clopton *et al.*, 1991) 6. (Rodriguez *et al.*, 2007) 7. (Schawang and Janovy, 2001) 8. (Hammerschmidt, 1838) 9. (Harry, 1967) 10. (Berndt, 1902) 11. (Kleespies *et al.*, 2008)
	Mattesia sp.[11] (n.i.a.)	n.i.a.			
	Steinina *S. ovalis*[2] (L)	n.i.a.			
Nematoda	***Heterorhabditis*** *H. bacteriophora*[1,2,3] (L) *H. floridensis*[3,4] (L) *H. georgiana*[4] (L) *H. indica*[3,4,5] (L) *H. mexicana*[4] (L) *H. zealandica*[6] (L) ***Oscheius*** *O. onirici*[7] (L) ***Pharyngodon*** spp.[8] (A) ***Physaloptera*** spp.[8] (A)	n.i.a.	Microscopic examination of haemocoel.	*Steinernema* spp. and *Heterorhabditis* spp. are reliant on high moisture content to infect. Infection can be prevented by proper diet storage in dry conditions.	1. (de Carvalho Barbosa Negrisoli *et al.*, 2013) 2. (Brown *et al.*, 2006) 3. (Shapiro-Ilan *et al.*, 2014) 4. (Shapiro-Ilan *et al.*, 2009) 5. (Shapiro-Ilan *et al.*, 2008)

	Steinernema *S. affine*[9] (L) *S. carpocapsae*[1,3,10] (L) *S. feltiae*[3,9] (L) *S. glaseri*[10] (L) *S. innovationi*[11] (L) *S. rarum*[1,4] (L) *S. riobrave*[1,3,4,5,10] (L)				6. (Lulamba and Serepa-Dlamini, 2020) 7. (Torrini *et al.*, 2015) 8. (Gałęcki and Sokół, 2019) 9. (Nielsen and Philipsen, 2004) 10. (Ramos-Rodriguez *et al.*, 2007) 11. (Ramakuwela *et al.*, 2018)
Viruses	Acheta domesticus densovirus (AdDV)[1] (L)	n.i.a.	Electron microscopy, CsCl density gradient centrifugation, and Sepharose CL 2B chromatography, confirmation with PCR.	n.i.a.	1. (Szelei *et al.*, 2011) 2. (Huger, 1969) 3. (Gencer *et al.*, 2020) 4. (Kelly *et al.*, 1979) 5. (Black *et al.*, 1981) 6. (La Fauce and Owens, 2008)
	Densovirus (E)[2] (L)	n.i.a.			
	Invertebrate iridescent virus6 (IIV6)[3] (L)	Paralysis of larvae observed after three days of infection			
	Invertebrate iridescent virus29 (IIV-29)[4,5] (P, A)	n.i.a.			
	Penaeus merguiensis densovirus (PmergDNV)[6] (L)	n.i.a.			

[a]n.i.a.: no information available.
[b]Life stages E: eggs; L: larvae; P: pupae; A: adults.

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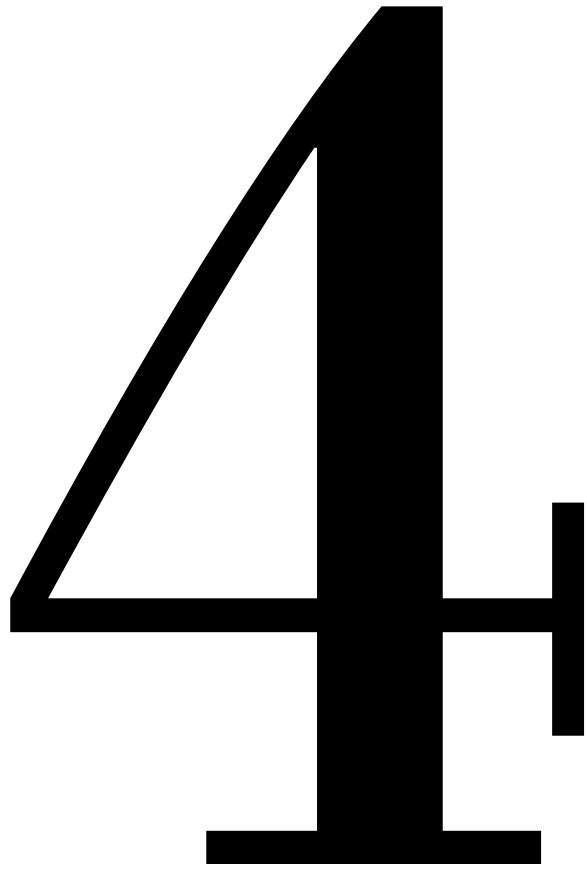

Chapter 4

Bacillus thuringiensis sv. *morrisoni biovar tenebrionis* impact and persistence in *Tenebrio molitor* larvae

Carlotta Savio, Pascal Herren, Agnès Rejasse, Joop J.A. van Loon, Christina Nielsen-Leroux

Abstract

The yellow mealworm (*Tenebrio molitor* (L.); Coleoptera: Tenebrionidae) has been proposed during the last decade as a suitable insect species for feed and food production. The inclusion of by- and side-products of cereal and vegetable production in the feed of yellow mealworm increases the probability of infection by *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* (Btt), an entomopathogen used in the biological control of coleopteran pest species. The lack of studies on effects of the spores and toxin forming Btt4AA1 strain on *T. molitor* larvae in mass-rearing systems led us to explore its effect on survival and growth of larvae and its persistence in larvae and frass. We exposed larvae of different body mass to a range of spore — crystal concentrations for free feeding on contaminated wheat bran for 72 hours. Susceptibility to the entomopathogen was dose dependent and directly correlated to larval body mass, larvae of higher mass being less susceptible. Growth and feed conversion ratio of survivors showed significant impact for low mass larvae while less or no effect was recorded for higher mass larva. Btt was still recovered from larvae and frass 14 days after feeding with non-contaminated wheat bran, indicating a certain level of persistence in the tested conditions and therefore a potential risk in *T. molitor* rearing.

Keywords
yellow mealworm, insect diseases, entomopathogen, mass-rearing, feed conversion ratio

1. Introduction

The yellow mealworm *Tenebrio molitor* L. (Coleoptera:Tenebrionidae) is a native European grain pest (Domínguez-Arrizabalaga et al., 2020). Nowadays it is mass-reared to meet the increasing global protein demands and the need to reduce the environmental impact of the food production chains (Smetana et al., 2021; van Huis, 2013; van Huis et al., 2021). The mealworms' high content of proteins of high nutritional value and their sustainability for inclusion in food has led to expansion of rearing companies in Europe (Stull et al., 2019; Smetana et al., 2021; Wade and Hoelle, 2020).

Several substrates are suitable as feed for the yellow mealworm, including the commonly used wheat bran and residual streams of cereal and vegetable production. Conversion of such residual streams from agricultural production by yellow mealworms contributes to the circularity of food chain (Kröncke and Benning, 2022). However, questions related to the safety of wastes and by-products used as insect feed substrates are emerging regarding insect, animal or human health in order to prevent diseases at the different levels of the food chain (European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2019 ; Maciel-Vergara et al., 2021; Oonincx et al., 2019).

In insect mass-rearing facilities, attention is both focused on insect pathogens and human and animal food-borne pathogens such as *Listeria monocytogenes* and *Salmonella* spp. (Mancini et al., 2019; Wynants et al., 2019).The microbial composition in mass-reared insects indicate in some studies both presence and persistence of some human pathogens (Vandeweyer et al., 2017). Indeed, *Tenebrio molitor* feed contamination studies showed a certain persistence of both *Salmonella enterica* (Wynants et al., 2019) and *Serratia marcescens* (Dupriez,et al. 2022). Therefore, insects can be vectors of various pathogens, for humans or animals and sub-optimal conditions in the mass-rearing system, in particular temperature, relative humidity, and insect density can favour the spread of entomopathogens (Cohen, 2018; Maciel-Vergara et al., 2021). The use of fungal and bacterial entomopathogens as biological control agents to suppress insect pests has a long history of use. Examples can be found in the application of bacteria such as *Bacillus thuringiensis* (Sanahuja et al., 2011) and fungi as *Beauveria bassiana* for protecting the crops from highly damaging pests as *Spodoptera frugiperda* L. (Lepidoptera: Noctuidae) (Jackson et al., 2010; Tozlu et al., 2022). *Bacillus thuringiensis* is an ubiquitous Gram-positive sporeforming bacterium that produces intracellular toxins, which accumulate as inclusions, called crystals, and spores during the stationary phase of its growth cycle (Domínguez-Arrizabalaga et al., 2020; Sanahuja et al., 2011; Schnepf et al., 1998), specific strains are pathogens for species in the order of Coleoptera such as *Tribolium castaneum* (L.)(Yılmaz et al., 2012; Younas et al., 2008).Other coleopterans as the yellow mealworm *Tenebrio molitor* could be infected by this entomopathogen if fed with cereal substrates (Kim et al., 2012). Sublethal effects of the infection on larval performance and survival can provide indications of a latent infection of the pathogen (Guo et al., 2022).

Former studies related to the virulence of *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* (*Btt*) on *T. molitor* neonates by Oppert et al. (2011) and Dean et al. (1996) applied force-feeding or free feeding of different larval stages and focused mainly on the mechanisms of action of the Cry3A toxin with larval gut receptors. In mass-rearing conditions infection takes place orally from the feed substrate. After entering the insect gut, ingested spores can germinate and the solubilized cry-toxin crystal, following enzyme processing, can cause disintegration of the mid-gut tissues. This will result in sublethal symptoms as inhibition of feed uptake and growth as reported for other *B. thuringiensis*-insect interactions (Raymond et

al., 2010, Nielsen-LeRoux et al., 2012). Subsequent tissue and organ failure may lead to death and bacterial proliferation in the cadaver resulting in a new cycle of spore crystal production and pathogen persistence as reported for the coleopteran species *Tribolium castaneum* (Milutinović et al., 2015).

Moreover, the presence of thermo- and UV- resistant biocontrol agents in the crops or stored grains, serving as feed for *T. molitor,* increases the risk of presence of higher toxin and spore concentrations in the feed provided to *T. molitor* (He et al., 2017; Oppert et al., 2011; Park et al., 2017).Therefore it is of interest to study *B. thuringiensis* sv. *morrisoni* biovar *tenebrionis* virulence and persistence in conditions that simulate the rearing system environment. This study aims to investigate on 1) the ability of *Bacillus thuringiensis* sv. *morrisoni biovar tenebrionis* to persist in the frass and in *T. molitor* alive larvae; 2) the impact of the entomopathogen to four *T. molitor* larval biomass classes as measured by the survival, the growth and larva feed conversion ratio. The outcome should increase the knowledge on the risk and impact of the presence of a coleopteran active *B. thuringiensis* strain in *T. molitor* rearing.

2. Material and methods

2.1. Insect rearing

Tenebrio molitor was obtained from the company Ynsect (Evry, France). Rearing and experiments took place in continuous darkness at 26°C and 60% RH. New generations were obtained by letting adults (20 couples) mate for 5 days on wheat bran (WB) in 750 ml plastic containers (Stackables, UK). *T. molitor* were reared on WB from new-born larvae throughout the whole life cycle and 1% water agar was provided twice a week as water supply.

2.2. Pathogen culturing and preparation of suspensions for inoculation

2.2.1. *Bacillus thuringiensis* culturing

Bacillus thuringiensis sv. *morrisoni* biovar *tenebrionis* isolate 4AA1 (Bacillus genetic stock center, Ohio State University, USA) cultures were grown in 1 L flasks on a rotary shaker at 200 rpm in 100 ml HCT medium (Lecadet and Martouret, 1967) for 96 hours. The bacterial cultures were then washed twice with 20 ml PBS with centrifugation step at 5000 rpm at T=4°C and a final dilution in 20ml PBS. The presence of spores and toxin crystals was verified by light microscopy at a magnification of x 1000 (Fig. 1). Total protein content was measured by Bradford analysis (Bradford, 1976) and SDS-PAGE 10% was applied to qualify and quantify the nature of the Cry3Aa toxin (65 kDa). Results allowed to quantify the toxin content expressed as µg/µl in the spore crystal toxin preparation. The total bacteria load (vegetative cells and spores) was determined by microbial enumeration (CFU=colony forming unit) on LBA (Luria Bertani Agar) plates after incubation at 30°C for 24 h. The heat resistant spore load was determined by using the same method after a thermal treatment at 82°C for 10 min.

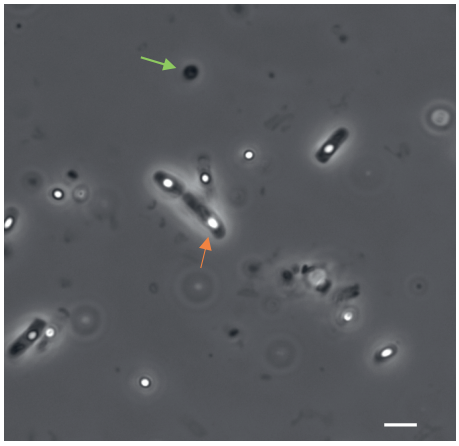

Figure 1. *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis*; toxin/crystal and spores indicated by respectively green and orange arrows; light microscopy image (magnification ≈1,000X, white bar =3 µm)

2.2.2. *Bacillus thuringiensis* infection suspension preparation

The concentration of toxin in the Btt4AA1 (spore/toxin) culture was used as base for preparing the infection suspensions and was verified by Bradford analysis. The suspension was processed with a high temperature treatment (82°C for 10 min) to inactivate Btt vegetative cells. The thermal treatment could modify the activity of the toxin, but its insecticidal activity is reported to be preserved (Lecadet and Martouret 1967). The spore load was determined by counting the number of colonies on LBA after an incubation period of 24 h at 30°C. Spore viability in the feed was verified by following the same protocol after incubating the mix of feed-Btt suspension at rearing environmental conditions for 3 days.

The concentration of toxin was expressed in µg toxin protein/mg feed. For example, to obtain an infection suspension of 0.1 µg toxin/mg feed, 200 µl of 0.1 µg toxin/µl suspension was homogenized in 200 mg feed. For a concentration of 1 µg toxin/mg feed, centrifugation of the Btt spore/toxin suspension was applied and the pellet was resuspended in sterile water before homogenizing in the feed. The amount (depending on the larval weight class) of feed assured the total consumption by larvae in the control treatment (feed containing sterile water) during 72 h. It was assumed that each larva in a cup with several individuals, consumed the same amount of feed.

2.3. Inoculation protocol

T. molitor larvae were inoculated by free feeding on pathogen-containing (spore-toxin) feed (Fig. 1). Environmental conditions were 26°C and 60% RH and continuously in the dark. Four different weight classes of *T. molitor* larvae were infected in 60 ml sterile cups (Labelians, Nemours, France) assuring a density of larval biomass of 0.15 g/cm^2 (example for 20mg larvae=120 larvae/cup). The wheat bran was inoculated with the spore-toxin preparation, see above, and several concentrations of alive spore and toxin crystal (Table 1). After 72 h of exposure to the Btt-containing feed, the insects were placed in clean cups and fed with wheat bran and water 1% agar for 11 days for recording Btt persistence in surviving larvae (only 30 mg larva).

Table 1. Overview of free-feeding infection assays of *Tenebrio molitor,* different larval weight classes, and *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* treatments, based on Btt Cry toxin concentrations with associated spores per mg wheat bran feed.

Treatment	Different Larval weight (mg)	Btt toxin (µg/mg feed)	Spores*/ mg feed
Btt_0	3, 5, 10, 30	Absent	0
Btt_0.1	5,10	0.1	5×10^5
Btt_0.25	3	0.25	1×10^6
Btt_0.3	5, 10	0.3	1.5×10^6
Btt_1	3, 10	1	5×10^6
Btt_1.5	30	1.5	6×10^6
Btt_2.5	3, 5, 30	2.5	7.7×10^6
Btt_5	3, 30	5	2×10^7

*The tested Btt spore-toxin preparations can come from different cultures

2.3.1. Assessing persistence of *Bacillus thuringiensis* in *Tenebrio molitor* larvae, frass and feed

Bacillus thuringiensis persistence in *T. molitor* larvae was investigated on 30 mg individuals every 2 days starting from day 3 (when larvae were placed on Btt free feed). Larvae and frass were separated with metal sieves (∅ 200 mm, pores=2mm, 1.4mm, 500 µm) (Retsch, Eragny sur Oise, France). Three larvae from each dose replicate were surface-sterilized with washing steps in sterile water – ethanol - sterile water, grinded with sterile pestles in 500 µl PBS (phosphate buffered saline pH 7.4) .Serial dilutions in PBS were plated on HCT (Hydrolysate of Casein Tryptone) agar (Lecadet et al., 1980). The enumeration of Btt colonies was done by CFU counting after 24 h of incubation at 30°C. Frass samples from the same assay were collected for enumerating Btt density in larval frass. A total of 20 mg frass from each cup was homogenized in 200 µl PBS and then serial dilutions were plated on HCT agar and incubated overnight at 30°C. The enumeration of the Btt colonies present in the feed was performed by sampling tree replicates of 20 mg of feed in 1 ml of PBS from the treatment Btt_0.5 and Btt_1 using the method applied for enumerating the bacteria in the larvae and frass.

2.3.2. Larval survival, weight gain and feed conversion

During the infection experiment, survival, growth and feed conversion ratio (FCR = total feed consumed divided by total larval mass gained, in mg fresh weight) were recorded gravimetrically with the analytical balance (Sartorius, BP211D, Goettingen, Germany, w=0.01/0.001g). FCR was recorded only for assays run with 5 and 10 mg larvae by dividing the total feed provided by the total mass gained per cup (Dupriez et al., 2022). Counting and weighing of larvae was recorded after mechanical separation with metal sieves from 3 technical

replicates at the beginning of the experiments, after the infections, and every two days during the remaining 11 days of the 14 days assay.

2.4. Data analysis

Counting of CFU colonies for determining Btt persistence in frass and larvae (only for the 30 mg ones) was performed in triplicate and data were analysed for normality (Q-Q plot distribution) followed by one-way ANOVA test and t-test for pair-wise comparison of treatments. Considering the larval survival and weight gain, every treatment was performed in 3 replicates, each having 30 up to 200 biological replicates (larva) depending on the larval stage. R 4.1 software was used for the Cox proportional hazard model to analyse survival of the larvae at day 14 and one-way ANOVA followed by Tukey HSD test were used to analyse the effect of Btt treatments on larval weight gain at day 14. The FCR data was analysed with one-way ANOVA test.

3. Results

3.1. *Bacillus thuringiensis* persistence in feed suspensions and *Tenebrio molitor* larvae and frass

The Btt spore counts in feed incubated for 72 h under the environmental conditions during the *in vivo* experiments showed no significant differences between the two initial Btt toxin concentrations (One way ANOVA, $p > 0.05$). No spores were found in the feed and therefore the difference was significantly lower than in the two Btt-spiked feed treatments ($t(5)=2.015$, $p < 0.0001$; Fig. 2).

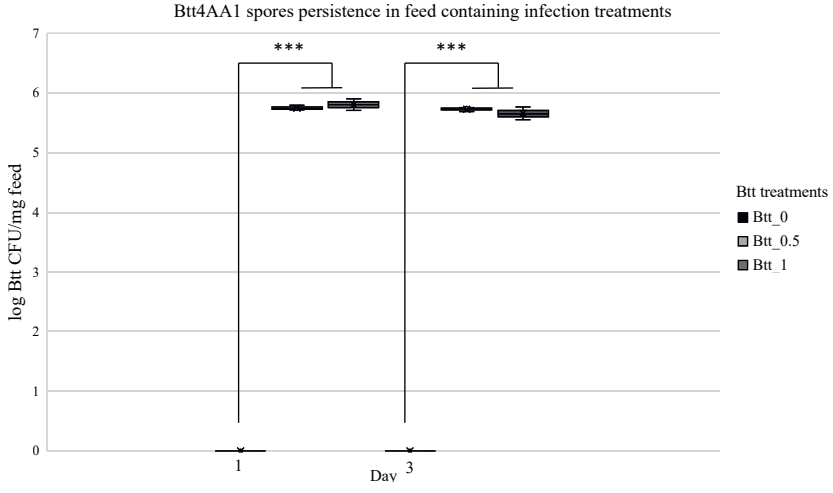

Figure 2. Btt spore counts (log CFU/mg feed) after 3 days of incubation at 26°C and 70%RH. Btt load was stable during the 3 days of infection. (Btt_0 = control treatment (water) Btt_0.5 = 0.5µg toxin/mg feed, Btt_1 = 1µg toxin/mg feed)

Bacillus thuringiensis was able to establish in surviving 30 mg *T. molitor* larvae fed with Btt_2.5 (1.5*10^8 Btt spores CFU/mg feed). Btt was found as spores at day 3 and was still present at day 14. From day 5 a portion of the bacteria was found as vegetative bacteria (total cell count is higher than spores only) (Fig.3). A small increase of total cells was found at day

5 and day 7 and all Btt was found as vegetative cells at the end of the experiment since no spores were recorded at day 14. Btt were detectable at concentrations of $2x10^4$ CFU/mg larvae 3 days after infection followed by a peak of $2x10^5$CFU/mg larvae at day 5 which decreased slightly to the initial value (Fig. 3). The one-way ANOVA test showed no significative difference between the total cell count for the spores for Btt_2.5 test during the assay, indicating the presence of only spores until the day 11. At day 14 only vegetative cells were detected, suggesting the ability of Btt to germinate in the larvae ($t(2) = 8.24$, $p = 0.038$).

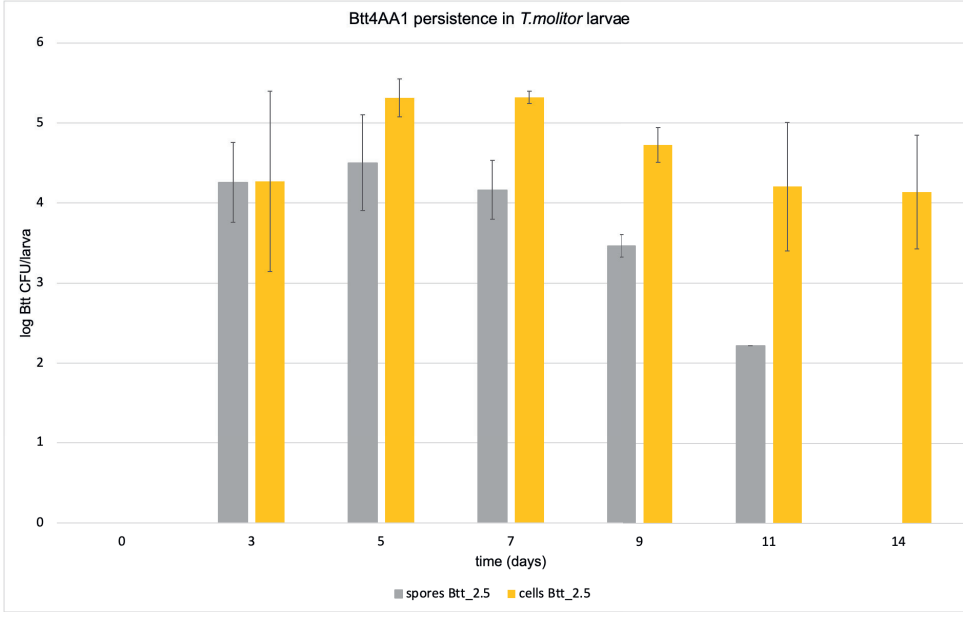

Figure 3. Number of *Btt* spores and total vital cells (spores in grey and total (spores and vegetative cells) in yellow) (log cfu/larva) in 30 mg *Tenebrio molitor* larvae during the remaining 11 days of the assay (one way ANOVA, $p>0.05$). Only vegetative cells were detected at day 14, no heat resistant spores were found.

The same method was applied for detecting *B. thuringiensis* in *T. molitor* frass. The presence of *Btt* in this case was highest at day 5 ($3x10^5$ cfu/mg frass) and decreased over time ($1x10^2$ cfu/mg frass), indicating that Btt was not able to develop further in 30 mg *T. molitor* larvae and in the frass when provided in single infection suspensions dosages of Btt_2.5 ($1.5x10^8$ Btt spores CFU/mg feed) and Btt_5 ($2.4x10^9$Btt spores CFU/mg feed) (Fig. 4). The low amount of Btt survived in the frass as spores as total count and spore counts were not significantly different.

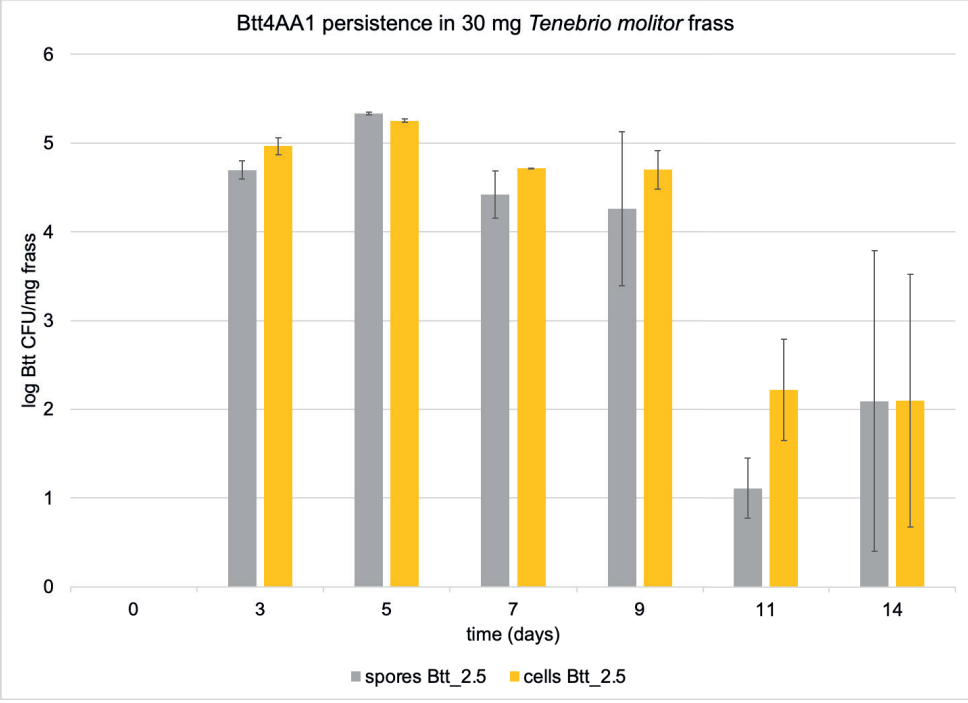

Figure 4. Presence of *Btt* in *Tenebrio molitor* frass (30mg larvae). Spores in grey and total cells (spores and vegetative in yellow) (log cfu/mg frass) during the 14 days of the assay. The Btt total vital and spore loads showed no statistical difference (one way ANOVA, p = 0.389).

3.2. Larval survival

Btt in the feed affected the survival of *T. molitor* (Fig. 5). Mortality was observed from day 3 following exposure to inoculated feed for all larval biomass classes. The concentration of Btt pathogen causing 50% of mortality (LC_{50}) for 3 mg larvae was Btt 5 µg toxin/mg feed (Btt_5; coxph, p = 1.38e-05) (Fig. 5 A). Larvae of 30 mg showed the highest resistance to Btt infection presenting no statistical significant trends in mortality responses to Btt_1.5 and Btt_2.5 treatments (p > 0.05). The highest concentration of Btt (Btt_5) was significantly different from the control Btt_0 resulting in 90% survival of 30 mg larvae (p = 0.0339) (Fig. 5 D). The treatments Btt_0.3 (p = 0.047), Btt_1.25 and Btt_2.5 presented a statistically significant effect on the survival of 5 mg larvae (p <0.0001). It was possible to observe a shift of the survival rate of 85, 75 and 70% on 5 mg larval class weight (Fig. 5 B). The effects of Btt_0.1, Btt_0.3 and Btt_1 treatment on the larval class weight of 10 mg were significantly different from Btt_0 even if presenting high survival values of 97.90% (Btt_0.01 p = 0.001; Btt_0.03 p = 0.00097; Btt_1 p = 0.00575) (Fig. 5 C).

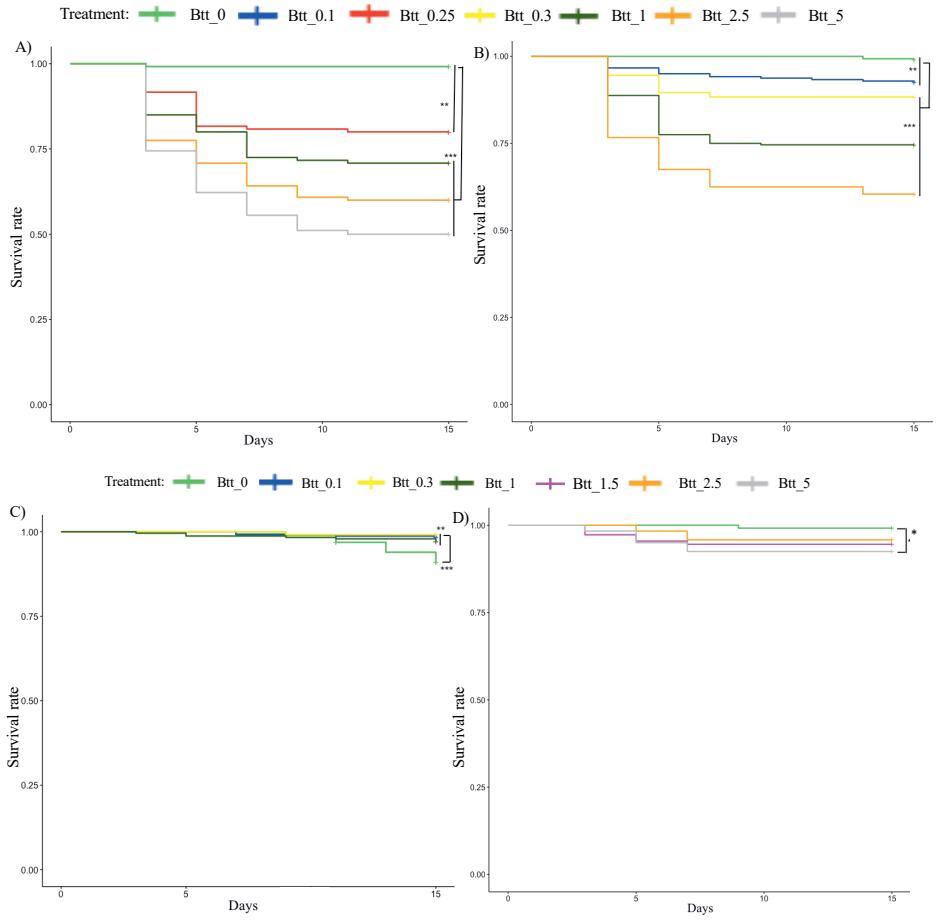

Figure 5. Kaplan-Meier curves showing survival during 14 days of *T. molitor* larvae with initial biomass (A) 3 mg; (B) 5 mg; (C) 10 mg; (D) 30-40 mg. Larvae were exposed to Btt4AA1 infection by free feeding on Btt inoculated wheat bran suspensions for 72h. The comparison of the results of the percentage of survival was made between *T. molitor* larvae populations from the same population larval size between each treatment and the control Btt_0 (*p < 0.01; **p < 0.001; *** p < 0.0001). (green = control treatment; blue = Btt_1; red = Btt_0.25; yellow = Btt_0.3; dark green = Btt_1; orange = Btt_2.5; grey=Btt_5)

3.2. Larval growth

The presence of Btt influenced the growth of *T. molitor* larvae and was measured at the end of the 2 weeks of experiment (Fig. 6). The Btt treatments resulted in a significant reduction in the growth of 3 mg-larvae after 14 days (One way ANOVA; $F(4,14) = 13,659$; $p < 0.0001$). Significant differences were found between treatment Btt_0 and Btt_0.25, Btt_1, Btt_2.5 and Btt_5 (Tukey's HSD; all comparisons $p < 0.001$).

Likewise a significant effect of the Btt treatments on growth of 5 mg-larvae was found (One way ANOVA; $F(2,5) = 45,969$; $p = 0.001$). Significant differences occurred between Btt_0 and the Btt_0.1 and Btt_2.5 treatments (Tukey HSD; for both comparisons $p = 0.012$) and between Btt_0.1 and Btt_2.5 ($p = 0.012$).

Growth of 10 mg-larvae was also significantly affected (One way ANOVA $F(3,4) = 127,586$; $p < 0.0001$). Significant differences were observed between Btt_0 and Btt_0.3 (Tukey HSD; $p = 0.019$), Btt_0 and Btt_1 ($p < 0.001$), Btt_0.1 and Btt_0.3 ($p = 0.005$), Btt_0.1 and Btt_1 ($p < 0.001$) and Btt_0.3 and Btt_1 ($p = 0.002$).
Growth of 30-40 mg larvae was significantly influenced by Btt-treatment (One-way ANOVA; $F(3,11) = 12,661$; $p = 0.002$). Treatments Btt_0 and Btt_1.5 (Tukey HSD; $p = 0.001$), Btt_0 and Btt_2.5 ($p = 0.012$) and Btt_0 and Btt_5 ($p = 0.048$) resulted in different growth rates.

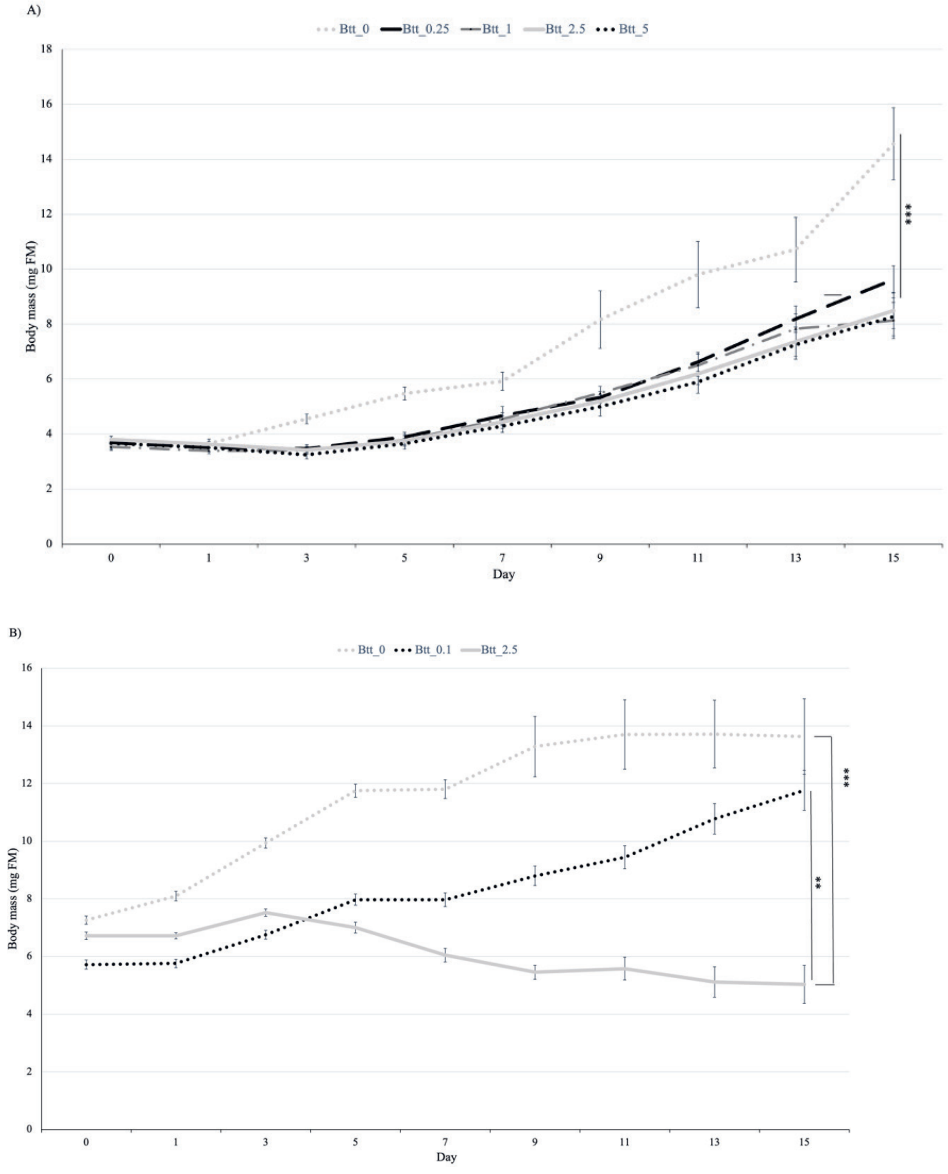

Figure 6. Growth curves of *T. molitor* larvae (mg FM,fresh matter) during the 14 days of assay for four classes of biomass at the start of the assay. A: ≈3 mg larvae; B: ≈5 mg larvae; C: ≈10 mg larvae and D: ≈30 mg.

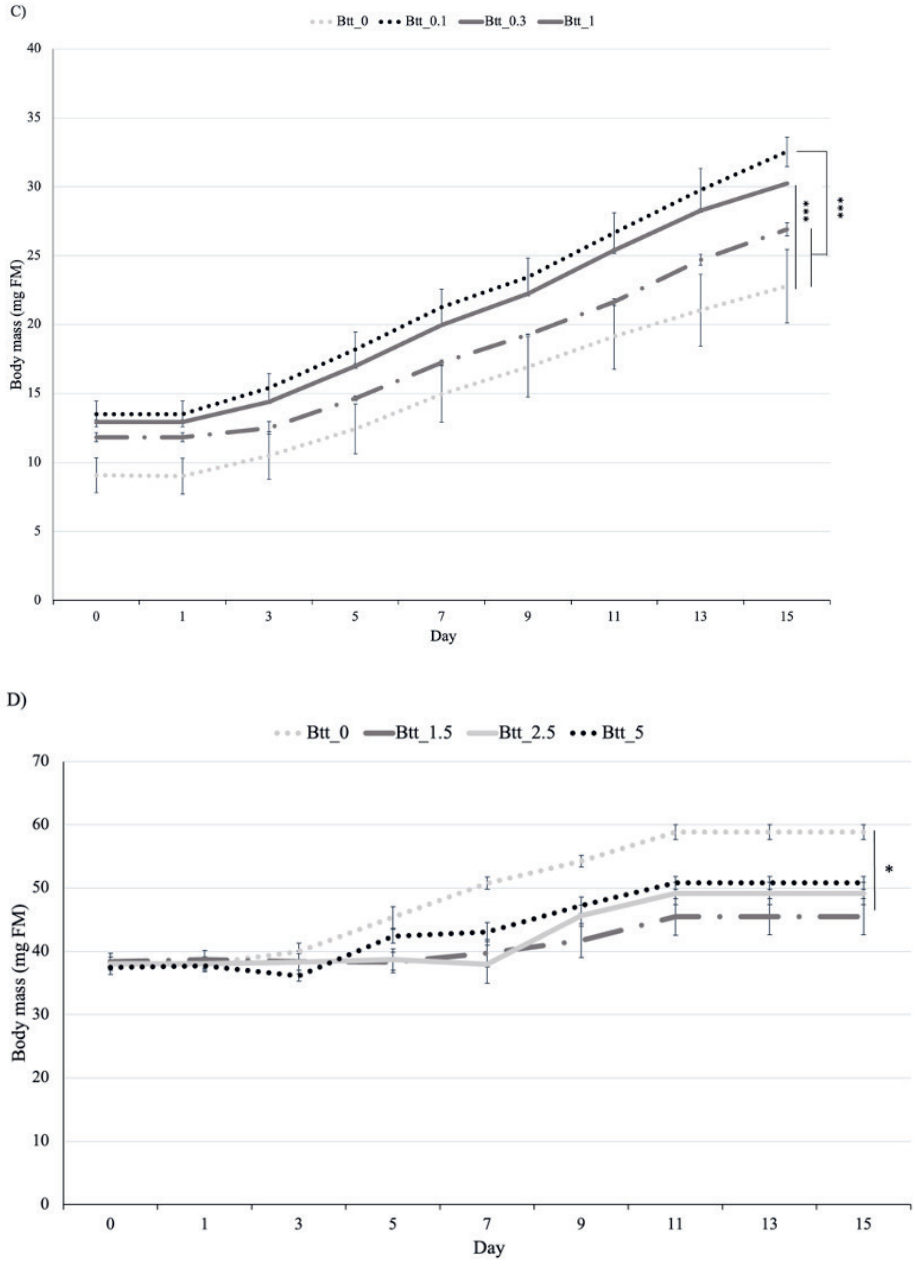

Figure 6. Growth curves of *T. molitor* larvae (mg FM,fresh matter) during the 14 days of assay for four classes of biomass at the start of the assay. A: ≈3 mg larvae; B: ≈5 mg larvae; C: ≈10 mg larvae and D: ≈30 mg.

The feed conversion ratio (FCR) measured after 14 days of assay of 5 mg-larvae exposed to Btt was not significantly influenced by the presence of the pathogen according to one way ANOVA test results for 5 mg larvae (One-way ANOVA; $F(3,4) = 1,227$, $p > 0.05$) in comparison to the Btt_0 treatment. The ability of 10 mg larvae to convert feed was reduced by the presence of the pathogen (One-way ANOVA; $F(3,4) = 52,8974$; $p = 0.00112$). Among all the treatments, only Btt_1 reduced significantly the FCR (t-test, $p < 0.01$) (Table 3).

Table 3. Feed conversion ratio (mean value and SD) of *Tenebrio molitor* larvae after two weeks since the start of Btt exposure during 72 h. Each infection treatment was replicated twice. Means having no superscript letter in common differ significantly. n.a. one replicate was excluded due to high mortality.

Initial larval weight	Infection treatments							
	Btt_0		Btt_0.1		Btt_0.3		Btt_1	
	FCR	SD	FCR	SD	FCR	SD	FCR	SD
5 mg	3.30[a]	0.26	6.60[a]	0.06	5.49 [a]	1.78	6.94	-[&]
10 mg	1.94[A]	0.05	2.04[A]	0.07	2.18[A]	0.07	2.66[B]	0.06

[&]SD could not be calculated due to high mortality in one replicate. The lower the FCR the better the conversion.

3. Discussion

The production of *B. thuringiensis* strains suitable for safe environmental release (Park et al., 2017) aiming at increasing the resistance of the Cry toxins to environmental stresses like high temperature, UV and desiccation (He et al., 2017; Oppert et al., 2011) may increase the risk of detecting the entomopathogens in cereal substrates provided as feed for the yellow mealworm. In the present study we searched to get knowledge on the impact of a Coleopteran active *B. thuringiensis* strain, the Btt4AA1 on *T. molitor* larva in conditions which might be found in mass rearing. Indeed, it is expected that Btt can be present in the wheat bran feed and therefore both the very small larva and larger ones may be exposed to the spores and toxin crystals. Therefore, our experiments explored different larval mass classes and various toxin/spore concentrations mixed with the wheat bran feed, to evaluate the relative susceptibility and impact on larval growth. The second issue addressed the persistence of Btt in the rearing environment in order to evaluate the potential risk.

Classical microbiological counting methods showed that Btt4AA1 provided by free feeding method could be sufficiently competitive for colonizing *T. molitor* gut as Btt vegetative cells were detectable after the provision of non-contaminated feed for 11 days after infection, showing the ability of the spores of the strain to germinate in *T. molitor* gut. However, further studies are needed for determining if longer periods of incubation would result in higher colonization rates or in complete clearance of the pathogen due to microbial competition in the gut or natural expulsion of the pathogen as result to peristaltic gut movement. Meanwhile in this study, persistence was only investigated in high mass larva and it might not be the case for lower mass larva. In addition, we found Btt in the *T. molitor* frass as spores only and even after having changed to new feed at least 4 times, suggesting that bacterial multiplication may not take place in the frass; but the UV and heat resistant spores can remain in the small dry frass particles. Further studies should explore the kinetics of total cell counts and spores /toxin

crystal in the cadavers of the dead larva in order to get information of Btt persistence in the rearing systems to estimate the potential risk of contamination. Indeed, laboratory studies with *Tribolium castaneum* indicated important multiplication ratio in cadavers (Milutinovic et al. 2015).

Our study shows that *T. molitor* performances were significatively impacted by the Btt infection, indeed both higher susceptibility in low larval body weight classes, in terms of decreased survival rate was recorded, and reduction of the larval growth in all tested weight classes in the presence of the pathogen was observed. The feed conversion ratio (FCR) was negatively affected by Btt infection in 10 mg larvae. The free feeding infection method could imply that the feed ingested by each individual was not homogeneous due to competitive feeding behaviors. Death only occurred in the individuals that ingested most spores and toxins while the ingested amount of Btt by the others was not sufficient for the pathogen to complete the infection and overcome the immune system responses (Vigneron et al., 2019). Meanwhile, our study shows that the mortality was directly related to Btt concentrations and inversely related to larval mass classes weight. Sublethal symptoms were observed with Btt infections, as a lower growth rate in the highest larval weight class. Oppert et al., 2011) observed a larval mortality after 30 days in larvae at Btt Cry3Aa dosage close to 1 µg/mg feed, comparable to the Btt_1 treatment applied in this study for infecting 3, 5 and 10 mg mealworm larvae. For these, different levels of susceptibility were observed, resulting in 75% survival for lower-class weight and 95% survival in the 10 mg class. Considering the growth of the individuals exposed to the same Btt_1 dosage, it is evident that the exposure to the pathogen results in a lower weight gain in the individuals at day 14 day in comparison to the controls, suggesting sublethal effects on survivors as previously suggested (Costa et al., 2000).

The Btt load and the weight gain was surprisingly not directly correlated for all mass classes. For example, 30 mg larvae, which did not die when infected with Btt_5 dosage gained higher weight than the population infected by Btt_1.5. This may suggest that Btt as vegetative bacteria (see Fig. 1) in its metabolically active form, can play a nutritional role for *T. molitor* (Vigneron et al., 2019). For the 10 mg control larva (Btt_0), the weight curve was lower than for the Btt treated ones, meanwhile this might be explained by the lower start weight for this group. Indeed, the mean mass gain during the 14 days was higher for the control than for thee Btt treated.

The density of the larvae could also play a role in increasing the resistance against pathogens, conferring to the larvae the so called “density dependent prophylaxis” characterized by increased immune system performances in high density conditions (Wilson and Reeson, 1998). Further research is needed to unravel connections between metabolic responses and immune pathways in relation to entomopathogens infections.

The 10 mg larvae showed a good FCR denoting an efficient feed transformation despite the presence of pathogen infection. The surface/biomass ratio of smaller individuals could explain the lower efficiency of the feed transformation in body mass (Oonincx et al., 2019; Byerly, 1967). Several factors influence the dynamics between insect and entomopathogen and some can reduce the sublethal symptoms and increase host survival. The microbiota composition of the insect could protect the host from pathogens infection by space and nutrient competition (Gupta and Nair, 2020) and by immune system activation (Dierking and Pita, 2020). Further research is needed to explore the effects of separated *Btt4AA1* toxins and spores on *Tenebrio molitor* larvae in mass reared conditions in order to understand the relative role and persistence of spores and crystals. Considering, microbial dynamics, the potential protective role played

by *T. molitor* symbionts and the diet composition against *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebronis* have still to be investigated.

4. Conclusions

In mass rearing conditions, the presence of the entomopathogen *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* affects *T. molitor* performance. Moreover, the study highlights the direct correlation between the weight class of the larvae and the susceptibility to Btt. Responses to Btt infection in terms of FCR and weight gain showed lower performances in presence of the pathogen. Btt dynamics in *T. molitor* larvae along the infection show the ability of Btt to persist in the alive larva. This may suggest a role of the mealworm gut microbiota in the protection against Btt when non-lethal doses are applied. In addition, it indicates a certain level of persistence and therefore a risk for infection of susceptible larval mass classes. Indicating the need to monitor the detection of Btt in the *T. molitor* rearing setup.

List of abbreviations

Tm: Tenebrio molitor; Btt: Bacillus thuringiensis sv. morrisoni biovar tenebrionis/Btt4AA1; WB: wheat bran, FCR: feed conversion ratio.

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Author contributions

Conceptualization: C.S., C.N.-L.; Writing original draft: C.S.; Writing – Review and editing: C.S., C.N.-L., J.J.A. v.L.; Visualization: C.S.; Project administration: C.S., C.N.-L.; Investigation: C.S., P.H., A.R.; Data curation: C.S., C.N.-L., J.J.A.v.L.; Resources: C.N.-L., J.J.A.v.L.; Funding acquisition: C.N.-L., J.J.A.v.L.

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Chapter 5

Impact of probiotics and egg white on *Tenebrio molitor* growth, microbial composition and pathogen infection

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Abstract

The industrial rearing for feed and food purposes of the yellow mealworm, *Tenebrio molitor,* on agricultural by-products may expose larvae and adults to entomopathogens used as biocontrol agents in crop production. Bacterial spores/toxins or fungal conidia from species such as *Bacillus thuringiensis* or *Metarhizium brunneum*, respectively, could affect the survival and growth of the insects. Therefore, the aim of this study was to investigate the potential benefits of a mealworm diet supplemented with probiotic bacteria or dried egg white, on larval development, survival and gut microbiome assemblage. Two probiotic bacterial species, *Pediococcus pentosaceus* KVL B19-01 and *Lactobacillus plantarum* WJB were added to wheat bran feed with and without dried egg-white as an additional protein source, directly from egg hatching. Larvae with a body mass of 20 mg were exposed during 72 h to *B. thuringiensis, M. brunneum* or their combination. Larval survival and growth were recorded for 14 days and the bacterial microbiota composition was analysed by 16S rDNA sequencing pre-pathogen exposure and at day 3 and 11 after inoculation with the pathogens. Increased growth and survival rate were observed for *T. molitor* larvae reared on feed supplemented with *P. pentosaceus*, conferring increased survival in case of co-infection. No significant impact of egg white on larval growth was recorded, while a minor effect of deactivated *Lb. plantatrum* was found in absence of pathogens. At day 14, the bacterial community composition of the larvae was similar in all treatments, indicating that the probiotic strain did not establish at a detectable level in the insect, however, its transient presence improved larval performance.

Keywords: yellow mealworm, insect diseases, entomopathogen, probiotics, insect health, *Bacillus thuringiensis*, *Lactobacillus plantarum, Metarhizium brunneum, Pediococcus pentosaceus*

1. Introduction

The predicted increased demand for protein is a driver to exploit several protein sources to preserve human health while mitigating the environmental impact of protein production (Belluco et al., 2013; Henchion et al., 2017; Smetana et al., 2016). Among the explored options, insects have been promoted by the Food and Agricultural Organisation of the United Nations (van Huis, 2013) as a promising protein source due to their nutritional values (van Huis et al., 2021) and their suitability for mass-rearing (Grau et al., 2017b). Among more than 2000 edible insect species (Jongema Y., 2019), the yellow mealworm (*Tenebrio molitor* L. (Coleoptera: Tenebrionidae) is one of the species suitable and authorised for feed and food production (European Commission, 2021). The larval life stage has between 11 and 19 instars (Ludvig, 1956) depending on environmental and nutritional conditions. These parameters influence also the chemical composition of the insect body leading to research to improve rearing conditions and diet composition to optimize protein production (Adámková et al., 2020).

In mass-rearing environments insects may be exposed to pathogens that could negatively affect their performance (Eilenberg et al., 2015). The source of these pathogens can be found in organic cereal products used as feed for the insects, as these products could contain biocontrol agents used during crop production or naturally present the environment, such as the entomopathogens *Bacillus thuringiensis* (Sanahuja et al., 2011) and *Metarhizium brunneum* (Russell et al., 2010).

While good manufacturing practices (GMP) and Hazard Analysis and Critical Control Point (HACCP) have been proposed for reducing the rate of infection and avoiding pathogen transmission (EFSA, 2015; EFSA Panel on Nutrition, Novel Foods and Food Allergens et al., 2021), several studies have also highlighted the important role of symbionts in maintaining host health (Brownlie and Johnson, 2009; Mueller and Sachs, 2015; Savio et al., 2022). Interactions between beneficial symbionts and insects from various orders have already proven to be effective in reducing pathogen incidence. For example, the honey bee *Apis mellifera* L. can benefit from hive supplementation of *Lactobacillus* spp. against infections by *Paenibacillus* larvae, the causal agent of the American foulbrood (AFB) disease. In this case, reduced mortality and pathogen loads suggest higher protection from pathogens by primed immunity from *Lactobacillus* (Daisley et al., 2020). The effects of prokaryotic probiotic strains have also been studied in insects reared for sterile insect technique programs, such as *Ceratis capitata* L. (Augustinos et al., 2015; Msaad Guerfali et al., 2021) as well as insects reared for food and feed such as *Hermetia illucens* L. (Franks et al., 2021; Kooienga et al., 2020;Yu et al., 2011) and *T. molitor* (Lecocq et al., 2021; Rizou et al., 2022).

Nonetheless, the role of the microbiota of *T. molitor* in maintaining health and the effects of the application of vital and deactivated potential probiotic strains on larval performance while exposed to pathogens are yet to be investigated. In this study, *T. molitor* larvae were reared on two diets, a plain wheatbran diet and a wheatbran diet enriched in protein in the form of dried egg white, each supplemented with two potential probiotic bacterial species, *Pediococcus pentosaceus* KVL B19-01 (Lecocq et al., 2021) and *Lactobacillus plantarum* WJB (Storelli et al., 2018), in their live and inactivated form to quantify larval performance and microbial community composition in rearing conditions mimicking the environment of mass-rearing systems.

The choice of the probiotic strains was driven by their origin (Lecocq et al., 2021; Savio et al., 2022), their previously tested efficacy on *T. molitor* larvae in laboratory rearing conditions (Lecocq et al., 2021) and on malnourished *Drosophila melanogaster* (Storelli et al., 2018), showing in both cases increased performance. Furthermore, we analysed whether the presence

of these probiotics could decrease the impact of two pathogens, the bacterium *B. thuringiensis* sv *morrisoni* biovar *tenebrionis* and the fungus *M. brunneum.* The bacterial gut microbiota composition was also monitored to assess the persistence of the probiotics in the larvae and its possible modulation due to the diet and the probiotics.

2. Materials and Methods

2.1. Preparation of probiotics and postbiotics

Pediococcus pentosaceus isolate KVL B19-01 was obtained from the University of Copenhagen (Department of Plant and Environmental Sciences, Denmark) and *Lactobacillus plantarum* isolate WJB was obtained from François Leulier, Ecole Normale Supérieure, Lyon, France) and were cultivated in Mann, Rogosa and Sharpe Broth (MRS)(De Man et al., 1960) for 24 h at 30°C respectively in anaerobic (oxygen <20%, closed jars with AnaeroGen 2.5 L bag, Oxoid, Thermofisher, France) and aerobic conditions. After washing the cultures, freeze-drying was performed at -85°C and 0.01 mbar for 48 h (Alpha 2-4 LSC bacis, Christ, DE). The freeze-dried bacteria were stored at -70°C. The postbiotic cells (deactivated cells) were obtained by heat treatment of the freeze-dried cultures at 121°C for 15 min in an autoclave. The efficiency of the freeze-drying and the deactivation process was verified by classical microbiological methods by enumerating the CFU of both strains on MRS agar after 24 h of incubation at 30°C.

2.2. Experimental diet preparation

Control diets for *T. molitor* larvae were composed of wheat bran or wheat bran and dried egg white (Louis Francois, Croissy Beaubourg, France) (mixed in ratio 24:1). The bacteria *P. pentosaceus* and *Lb. plantarum* were respectively added to the control diets to obtain a load of 10^9 CFU/mg diet (50 mg diet and 0.5 mg freeze-dried bacteria). The same amount of deactivated freeze dried bacteria was added to the two control diets following the protocol described by Lecocq et al. (2021).

Table 1. Composition of the experimental diets provided to *T. molitor* larvae during the assay.

	Experimental diets									
	WB	WB_Ppp	WB_Ppt	WB_Lp	WB_Lbt	WE	WE_Pp	WE_Ppt	WE_Lp	WE_Lpt
Ingredient (%)										
Wheat bran (WB)	100	100	100	100	100	90	90	90	90	90
Dried egg white (WE)						10	10	10	10	10
Pediococcus pentosaceus KVL B19-01 (Pp)		X					X			
P. pentosaceus KVL B19-01 deactivated (Pp_t)			X					X		
Lactobacillus plantarum WJB (Lb)				X					X	
Lb. plantarum WBJ deactivated (Lb_t)					X					X

2.3. *Tenebrio molitor* rearing and probiotic provision

Tenebrio molitor larvae were sourced from the private company YNSECT (Evry, France). Adult beetles (20 males and 20 females; >7 days after eclosion) were added to 750 ml plastic boxes (15 cm x 9.5 cm = 142.5 cm^2; Stackables, UK) containing 50 g of one of the 10 experimental diets (see Table 1). After 5 days, the adults were removed and the hatching larvae received 10 g water agar (1% w/v) twice a week starting one week after removal of the adults. The pathogen infection experiments started when the individuals reached a body mass of 20 mg (Fig. 1). The individual larval weight was recorded gravimetrically (Sartorius, BP211D, Goettingen, Germany, 1 mg precision). The rearing took place at at 28°C in complete darkness. Water containers were added at the bottom of the incubator to reach a relative humidity of *ca*. 65%.

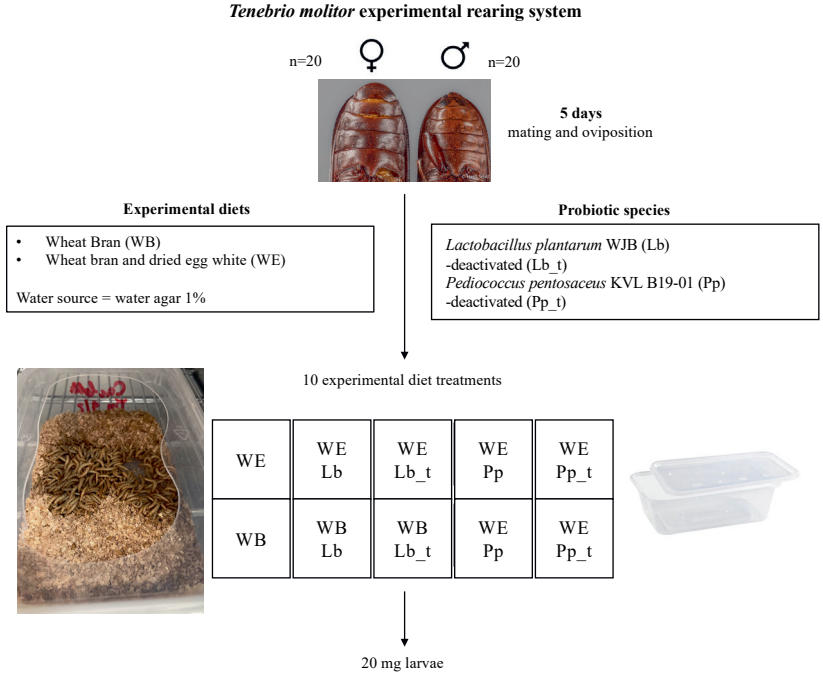


Figure 1. *Tenebrio molitor* experimental rearing system. Adults mate on wheat bran (WB) or dried egg white and wheat bran diet (WE) diet supplemented with the selected probiotic species *Lb. plantarum* WJB (Lb) or *P. pentosaceus* KVL B19-01 (Pp) freeze dried in their vital or deactivated (indicated by 't') form.

2.4. Culturing of bacteria and preparation of suspension for pathogen infection

2.4.1 Bacillus thuringiensis

Bacillus thuringiensis sv morrisoni biovar tenebrionis isolate 4AA1 (Btt) (*Bacillus* genetic stock center, Ohio State University, USA) was grown in HCT medium (5 g tryptone, 2 g bacto

casamino acids, 6.8 g KH_2PO_4, 0.1 g $MgSO_4$, 0.002 g $MnSO_4$, 0.014 g $ZnSO_4$, 0.15 g $CaCl_2$ and 0.022 g ammonium ferric citrate in 1 l dH_2O) (Lecadet et al., 1980) for 96 h at 30°C 200 rpm. The bacterial cultures were then washed twice with 20 ml PBS with centrifugation step at 15000 rpm at 4°C and a final dilution in 20 ml PBS. The presence of spores and crystal toxins was verified by optical microscopy observation. Bradford protein analysis (Bradford, 1976) using Biorad Microprotein assay was performed for quantifying the protein content of the Btt crystal toxin and SDS-PAGE 10% was used to qualify their nature (relative part of the Cry3Aa 65kDa toxin). Results were used to calculate the toxin content of *Btt* culture in µg/µl. The total bacterial load was determined by classical microbiological methods by enumerating the CFU on LBA (Luria Bertani Agar) at 30°C for 24 h, with serial dilutions of the washed and concentrated spore - crystal suspensions. The spore load was determined by using the same method after a thermal treatment on the culture at 80°C for 10 min.

Taking into consideration the results of the Bradford analysis, specific concentrations of toxin were prepared. The ratio of toxin spore suspension in the feed during the assay was expressed in µg toxin/mg feed and the volume of suspension added in the feed was 100 µl/100 mg feed.

The infection suspension was exposed to high temperature treatment (80°C) for 10 min to deactivate vegetative cells in order to provide the insects with Btt spores only. The spore load was then determined by CFU counting on LBA medium after an incubation period of 24 h at 30°C. The Btt toxin concentration used for the infection was prepared by concentrating the infection suspension in order to obtain a final load of 2.5 ug toxin/mg feed and $2x10^7$ spores CFU/mg.

2.4.2. Metarhizium brunneum

Metarhizium brunneum isolate KVL12-30 (Department of Plant and Environmental Sciences, University of Copenhagen) was grown on Petri dishes (9 cm diameter, triple vented) containing Sabourand Dextrose Aagar (SDA) medium (65 g/l) at 23°C in complete darkness for 21 days. The conidia were harvested with a Drigalski spatula by pouring 10 ml of Tween20 (0.05%) in the Petri dishes. Two washing steps with 15 ml of Tween20 (0.05%) with a centrifuge (3,000 rpm for 3 min) for discarding the supernatant each time were followed by a dilution step with water to obtain a 1,000 times diluted suspension. From the latter suspension 20 µl was added to a 0.2 mm Fuchs-Rosendahl hemocytometer and conidia were counted under a light microscope at 400x magnification. The conidia were counted in four squares (one square containing 16 cells) diagonally starting on the lower left corner. The concentration (conidia/ml) was then calculated by multiplying the average number of conidia per square with 5,000,000/ml (1,000 times dilution / 0.0002 ml volume). The spore suspensions were then prepared from the stock suspension using the formula C1*V1=C2*V2 (C = concentration, V = volume). All spore suspensions were prepared and used for inoculation on the same day. A germination test was performed to assure the ability of the fungal conidia to germinate. On the day of the assay, 100 µl of infection suspension were plated on SDA medium and incubated for 24 h at 26°C at 65% RH in the dark. Conidia germination was recorded by calculating the % of conidia/Petri dish in 3 Petri dishes. Values of conidia germination above 99% were accepted, meaning that the fungi were able to germinate in the conditions of infection. The *M. brunneum* conidia suspension used for the infection was 30,000 conidia/mg diet.

2.5. *Tenebrio molitor* co-infection assay

Tenebrio molitor larvae were infected with *Bacillus thuringiensis sv morrisoni biovar tenebrionis* 4AA1 (*Btt*) and *Metarhizium brunneum* KVL 12-30. The pathogens were provided

in the diet singly or in combination to investigate effects of co-infection on *T. molitor* larval performance and effects on larval microbial community composition.

2.5.2. Inoculation of Tenebrio molitor and subsequent actions

Larvae of *T. molitor* with a bodymass of 20 mg from the 10 different treatments were selected for infection studies. Groups of 20 larvae were placed in sterile plastic cups (60 ml, Qualibact® resistant 95 kPa, Labelians, France) to obtain a density of 0.07 g/cm^2 without feed for 24 h (Fig. 2) in four replicates for each infection treatment. An amount of 100 mg of diet, composed of wheat bran and egg white (10%) (WE) or wheat bran (WB) were poured into each cup. A volume of 100 µl of Btt suspension or *M. brunneum* suspension or 50 µl Btt +50 µl *M. brunneum* suspension or 100 µl sterile water as negative control treatment (1µl/mg) were poured in the feed for inoculation. A wet paper was inserted in the cups for increasing the humidity. Then the cups were covered by a paper tissue and incubated at 28°C for 72 h.

After 72 h, the dead insects, frass, and remaining feed were mechanically separated by sieving (∅ 200 mm, pores sizes 2 mm, 1.4 mm and 500 µm) (Retsch, Eragny sur Oise, France). The larvae (about 400 mg larval biomass) were then transferred to new sterile cups containing WB diet (30% of the larval biomass (≈400/100x30)) and water agar 1% ((≈400/100x60) 60% of the larval biomass). The number of surviving larvae and the weight of all larvae in the cup were subsequently recorded every 2 days for 11 days.

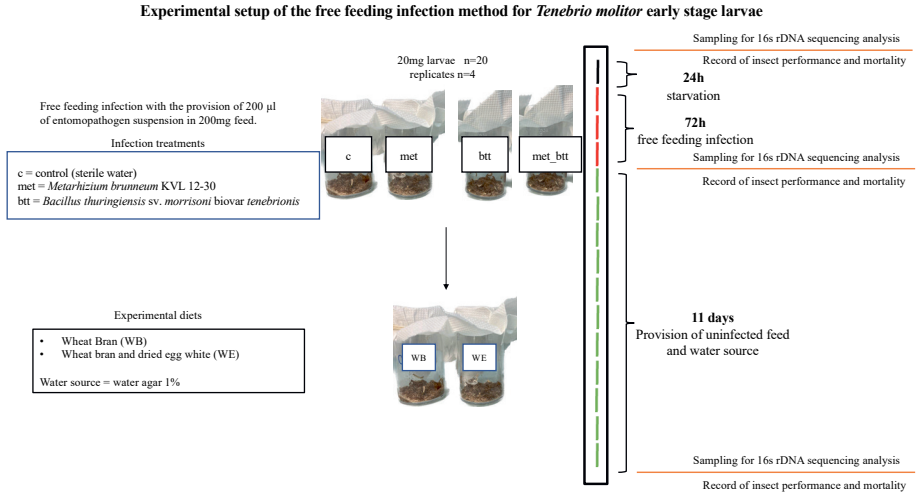


Figure 2. Experimental setup of the inoculation of *Tenebrio molitor* larvae with the entomopathogens *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* (btt), *M. brunneum* KVL 12-30 (met) or both pathogens (met_btt). After 24 h of starvation (black line), the larvae were exposed to the entomopathogens or sterile water (c) by free feeding method for 72 h (red lines), followed by 11 days of provision of uninfected feed (WE or WB diet) and water agar every 2 days (green lines).

2.5.3. Statistical analysis

Statistical analysis of larval survival, relative growth and the larval individual mean mass (IMM) were performed with R 4.2.2 R Core Team, 2022). Each of these variables were modelled as functions of probiotic, diet, pathogen, and their interactions.

A generalized linear model with a binomial error distribution was used to model the number of surviving larvae. Given that one of the categories presented complete separation (i.e., all larvae survived) a bias reduction method (brglmFit) from the R package brglm2 (Kosmidis et al., 2022) was used. Wald tests were then used to assess statistical significance of factor effects with the package lmtest. Thereafter, estimated marginal means and contrasts (package emmeans; Lenth et al., 2023) were used to compare the differences between the different treatments and the control category (no pathogen and no probiotics) within a given diet and pathogen inoculation category. P values were adjusted by an approximation of Dunnett's method. Similarly, contrasts were used to estimate the differences in survival between diets with the same probiotic and pathogen status.

The IMM was calculated by dividing the biomass of the alive larvae at the end of the assay by total number of the larvae and the relative growth was recorded by dividing the difference between the final and the initial weight of the population (all larva per cup) by the initial weight following the methods previously used by Dupriez et al. (2022). Analysis of variance was used to test the relative growth and the statistical significance of factor effects on IMM. Thereafter, estimated marginal means and contrasts were used as for survival. P values for contrasts between treatmentswere adjusted with the Tukey method.

2.6. Microbiota composition

Sequencing of 16S rDNA was performed for exploring the ability of the probiotics to persist in the gut of *T. molitor* and their effects on larval microbiota composition in the presence and absence of pathogen infection and the impact of the diet.

2.6.2. DNA extraction

Larvae were sampled from each treatment when an individual body mass of 20 mg was reached (prior to pathogen exposure), 72 h after pathogen infection treatments (day 3) and 11 days after the pathogens' removal (day 14). DNA extraction was performed on 4 technical replicate samples composed of 3 individuals each. The larvae were surface sterilized by 3 washing steps consisting of successive immersion in diethylpyrocarbonate (DEPC) water, EtOH 70% and DEPC water for 30 s. Three larvae were pooled, grinded with liquid nitrogen and DNA extraction was performed with DNeasy® PowerSoil® Pro Kit (Qiagen®). The quality of the genetic material was determined by Nanodrop and Qubit analysis. Samples were stored at -80°C.

2.6.2. 16S rDNA sequencing analysis

The V3-V4 hyper-variable regions of the the ribosomal RNA subunit 16S rDNA gene were amplified from the DNA extracts during the first PCR step using universal primers PCR1F_343 and PCR1_R784 (Table 2) which are fusion primers as described by Nadkarni et al. (2002). This PCR was performed using 2 U of a DNA-free Taq DNA Polymerase and 1x Taq DNA polymerase buffer (MTP Taq DNA Polymerase, Sigma-Aldrich, USA). The buffer was completed with 10 nmol of dNTP mixture (Sigma-Aldrich, USA), 15 nmol of each primer (Eurofins) and Nuclease-free water (Qiagen, Germany) in a final volume of 50 µl.

The PCR reaction was carried out in a T100 Thermal cycler (Biorad, USA) as follows: an initial denaturation step (94°C for 10 min) was followed by 30 cycles of amplification (94°C for 1 min, 68°C for 1 min and 72°C for 1 min) and a final elongation step at 72°C for 10 min.

Amplicons were then purified using a magnetic beads CleanPCR (Clean NA, GC Biotech B.V., the Nederlands) in a 96 well format. The concentration of the purified amplicons was controlled using a Nanodrop spectrophotometer (Thermo Scientific, USA) and a subset of amplicons size was controlled on a Fragment Analyzer (AATI, USA) with the reagent kit ADNdb 910 (35-1,500 bp).

Sample multiplexing was performed on the Abridge Platform (NRAE, Jouy en Josas, France) by adding tailor-made 6 bp unique indexes during the second PCR step at the same time as the second part of the P5/P7 adapters to obtain primers PCR2_P7F and reverse primer PCR2_P7R (Table 2). This second PCR step was performed on 50–200 ng of purified amplicons from the first PCR using 2.5 U of a DNA free Taq DNA Polymerase and 1xTaq DNA polymerase buffer. The buffer was completed with 10 nmol of dNTP mixture (Sigma-Aldrich, USA), 25 nmol of each primer (Eurofins, Luxembourg) and Nuclease-free water (Qiagen, Germany) up to a final volume of 50 µl. The PCR reaction was carried out on a T100 Thermal cycler with an initial denaturation step (94°C for 10 min), 12 cycles of amplification (94°C for 1 min, 65°C for 1 min and 72°C for 1 min) and a final elongation step at 72°C for 10 min. Amplicons were purified as described for the first PCR reaction. The concentration of the purified amplicons was measured using Nanodrop spectrophotometer (Thermo Scientific, USA) and the quality of a subset of amplicons (12 samples per sequencing run) was controlled on a Fragment Analyzer (AATI, USA) with the reagent kit ADNdb 910 (35-1,500 bp). Controls were carried out to ensure that the high number of PCR cycles (35 cycles for PCR 1 + 12 cycles for PCR2) did not create significant amounts of PCR chimerae or other artifacts. The region of the 16S rDNA gene to be sequenced has a length of 467 bp for a total amplicon length of 522 bp after PCR 1 and of 588 bp after PCR 2 (using the 16S rDNA gene of *E. coli* as a reference).

Negative controls to assess technical background were included using nuclease-free water (Qiagen, Germany) in place of the extracted DNA during the library preparation.

All libraries were pooled with equal amounts to generate equivalent numbers of raw reads for each library. The DNA concentration of the pool (no dilution, diluted 10x and 25x in EB + Tween 0.5% buffer) was quantified on a Qubit Fluorometer (Thermofisher Scientific, USA). The pool, at a final concentration between 5 and 20 nM, was used for sequencing.

Table 2. List of primers used for running the PCR1 and PCR2 step of 16S rDNA sequencing analysis.

Primer name	Primer sequence (5'-3')	rRNA operon binding site
PCR1F_343	CTTTCCCTACACGACGCTCTTCCGATCT-ACGGRAGGCAGCAG partial P5 adapter–primer	V3-V4
PCR1_R784	GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT partial P7 adapter–primer	V3-V4

PCR2_P5F	AATGATACGGCGACCACCGAGATCTACACT-CTTTCCCTACACGAC partial P5 adapter–primer targeting primer 1F	
PCR2_P7R	CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTGACT-GGAGTTCAGACGTGT partial P7 adapter including index–primer targeting primer 1R	

2.6.3. Sequencing

The pool was denatured (NaOH 0.1N) and diluted to 7 pM. The PhiX Control v3 (Illumina, USA) was added to the pool at 15% of the final concentration as described in the Illumina procedure. 600 µl of this pool and PhiX mixture were loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions using MiSeq Reagent Kit v3 (2x300 bp Paired-End Reads, 15 Gb output). FastQ files were generated at the end of the run (MiSeq Reporter software, Illumina, USA) to perform the quality control. The quality of the run was checked internally using PhiX Control and then each paired-end sequence was assigned to its sample using the multiplexing index.

2.6.4. Bioinformatic analysis

Resulting sequences were received in the format of de-multiplexed FASTQ file and analysed by using FROG (Version 4.0.1) (Afgan et al., 2018). The sequences obtained from the 267 samples were analysed according to the following workflow: pre-processing (maximum read size 250, maximum rate of mismatch in the overlap region = 0.1, Vsearch software to merge paired-end reads, 150-450 limits for amplicon sizes, primers 5'ACGGRAGGCAGCAG and 3' AGGATTAGATACCCTGGTA), swarm clustering (distance = 1), chimera removal, OTU filtering (Minimum OTU abundancy as proportion or count = 0.00005, recommended by Bokulich et al.(2013), to keep OTU with at least 0.005% of all sequences, phiX database for contaminants). Affiliation by blast (Version 2.10) with the reference DATABASE SILVA 138.1. Chao1, Shannon and Simpson index were used for determining the Alpha-diversity. Beta-diversity was determined with the weighted unifrac method and NMDS plot. A permutational multivariate ANOVA using distance matrices (vegan::adonis) was performed to determine whether the separation of the groups was significant.

3. Results

3.1. Larval performance

3.1.1. Survival

A model that included the 2nd and 3rd order interactions improved the model fit over a model containing only the main effects (Chisq = 50.148, df = 31, p = 0.01619). Survival of *T. molitor* larvae 11 days after the probiotic provision treatments ended showed differences. In case of WB reared larvae, the provision of *P. pentosaceus* KVL B19-01 vital (Pp) and deactivated (Pp_t) determined increased survival (92.6 and 94% respectively) in case of co-infection (met_btt) in comparison to the larvae reared on WB control diet and exposed to the same

pathogen infection (74.6%) (Pp: p = 0.0415; Pp_t: p = 0.0227; Fig. 3). No effect of *Lb. plantarum* was found on survival in either infected or uninfected larvae provided with WB or WE (P > 0.05).

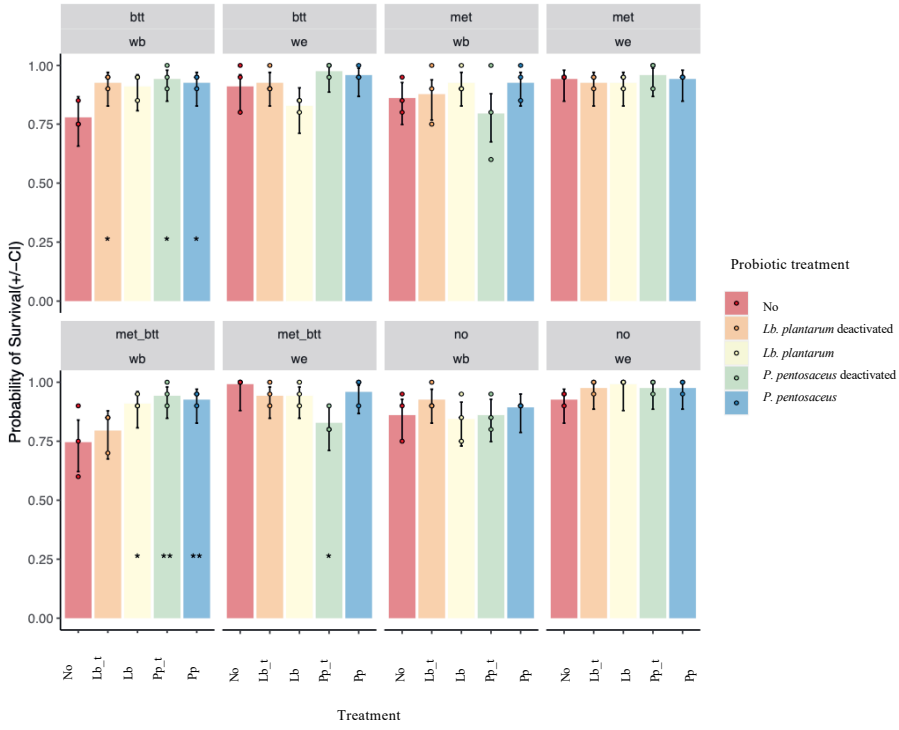


Figure 3. Probability of survival (± 95% confidence interval) of *Tenebrio molitor* larvae after 2 weeks of assay. Treatments were the presence in the diet of the active probiotic strains (*P. pentosaceus,* Pp and *Lb. plantarum* Lb) and inactivated (*P. pentosaceus* Pp_t and *Lb. plantarum* Lb_t) on the survival of *Tenebrio molitor* larvae fed with wheatbran (WB) diet or wheatbran + eggwhite diet (WE) and infected with *Bacillus thuringiensis* (btt), *M. brunneum* (met) or both pathogens (met_btt). Each treatment is the mean of three biological replicates of groups of 20 larvae each. The addition of *P. pentosaceus* either live or deactivated resulted in a significantly higher survival of larvae fed with WB and in co-infection conditions (met_btt)(Pp: p = 0.0415; Pp_t: p = 0.0227).

3.1.2. Larval performance

The relative growth (weight gain between day 3 and day 14) in the control compared to the other treatments during the assay was significantly influenced by the presence of the pathogens and not by diet composition or by probiotic addition (ANOVA, Type III test, pathogens p = 0.003, diet p = 0.117, probiotic p = 0.137). Significant differences were observed between the co-infection met_btt and the control (p = 0.003) as well as between met_btt and btt (p = 0.033). Similarly, there were differences between the met_btt co-infection and met infection (p = 0.045). No significant differences were detected between the control and Btt infection (p = 0.84) or the control and met infection (p = 0.78) or between the Btt and met infections (p = 0.99).

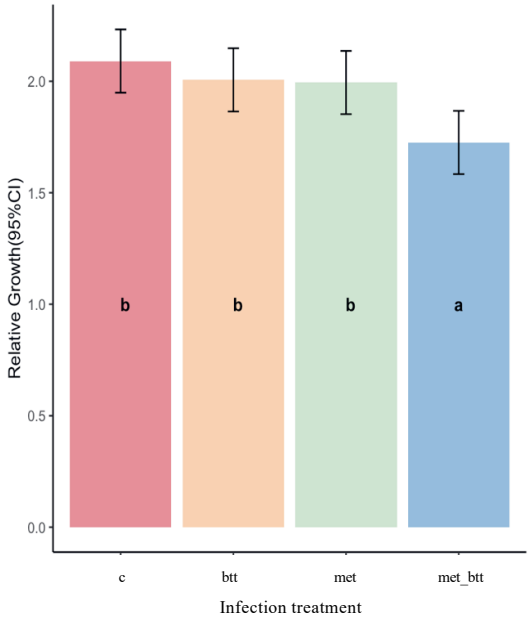


Figure 4. Effect of pathogens on the relative growth of *Tenebrio molitor* larvae after 2 weeks of assay. Single infections (btt = *Bacillus thuringiensis sv morrisoni biovar tenebrionis*; met = *Metarhizium brunneum*) had no significant impacts on relative growth (btt p = 0.84, met p = 0.78). Co-infection (met_btt) resulted in significantly lower relative growth (p = 0.003) after 2 weeks. Bars show estimated marginal means.

The individual mean mass IMM was impacted by the interaction of probiotics, diet and pathogens ($F_{12,80} = 1.81$, p = 0.06. Larvae fed with WB diet supplemented with deactivated *Lb. plantarum* (Lb_t) and infected with with *M. brunneum* (met) and the ones fed with WB diet supplemented with vital *Lb. plantarum* (Lb) and infected with both pathogens (met_btt) presented a higher IMM than the WB control (p = 0.038 and p = 0.05 respectively), indicating that the addition of the probiotic reduced the negative impact of the pathogens on larval growth.

Overall, no strong significant effects of *P. pentosaceus* or *Lb. plantarum* on IMM of *T. molitor* larvae after 2 weeks in single infection conditions were found (p > 0.05). Meanwhile, a small significant effect of live probiotics added to the diet was found (ANOVA Type III test, p = 0.048) in case of co-infection. Indeed, a significant effect of *Lb. plantarum* deactivated (Lb_t) on co-infected larvae (met_btt) fed with WB was found (p = 0.038) (Fig. 5). The P values were adjusted by dunnettx method for 4 tests.

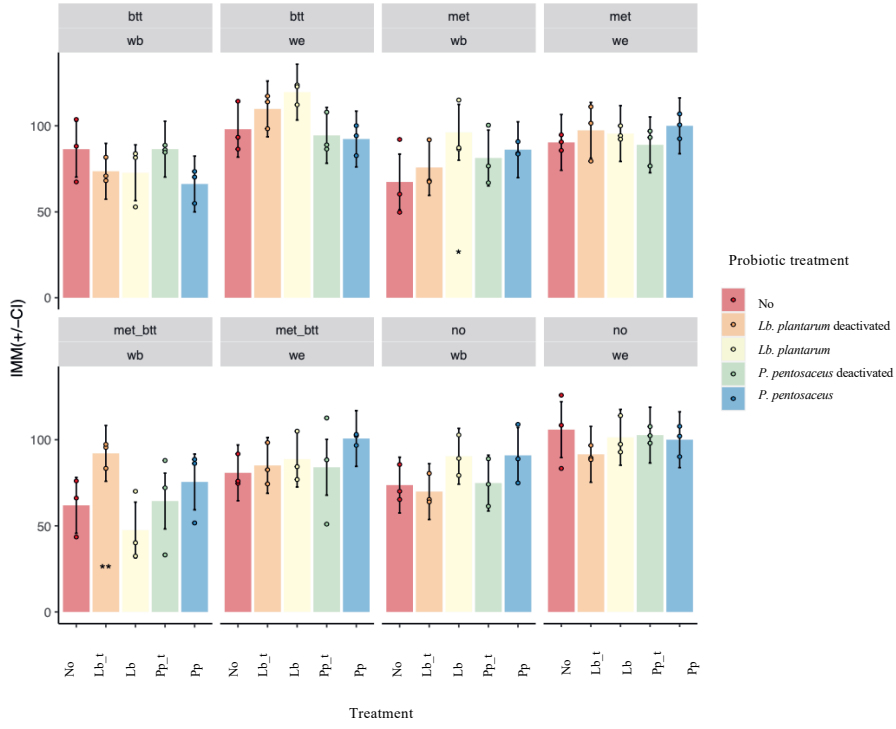


Figure 5. Individual mean mass (IMM) of *T. molitor* larvae after 2 weeks of growth. *Lb. plantarum* deactivated (Lb_t) was the only treatment resulting in an effect on co-infected larvae (met_btt) fed with WB ($p = 0.038$). The other treatments showed no significant effects on IMM ($p > 0.05$).

3.2.Microbial community composition

16S rDNA-based bacterial composition of *T. molitor* larvae provided with probiotics

The effects of probiotic treatments on the bacterial community composition have been investigated in the two diets (WB and WE) from individuals exposed to different treatments (probiotics, pathogens). The aim was to identify to what level the diets and the presence of probiotics and pathogens modify the bacterial composition and the OTU abundance over time after the removal of the treatments. Key interest was also to know to what extent the probiotics or the pathogen could persist in the larvae. The analyses were performed on larvae from day 0 (just after removal from diet with probiotics), from day 3 (just after removal from the diet with pathogens and at day 14, corresponding to 11 days fed with WB or WE. All data were compared with control larvae fed only with WB or WE throughout the whole period from egg to the 14 days assay (Fig. 1 and 2).

The 16S sequencing Illumina analysis resulted in a total of 12890914 Nb sequences. Pre-processing allowed to select a number of pre-paired sequences of 1849872 Nb (14.35%). After chimera removal (27.5%), the OTU filter processing kept 9841546 (92.7%) sequences.

Taxonomy was assigned with database SILVA 138.3 (McLaren, Michael R. and Callahan, Benjamin J., 2021) determining the affiliation of 100% sequences with 0.005% sequence

abundance and an identity of acceptance of 97%, a total of 390 OTUs were obtained from 275 samples and were classified into 6 phyla, 8 classes, 26 orders, 41 families, 85 genera and 124 species (Table S1).

At day 0, the microbial composition was mainly represented by *Enterococcus* (relative % abundance in WE control= 39, WE-Lp = 6, WE-Pp = 23 , WE-Lb_t = 0, WE-Pp_t = 6), *Lactococcus* (RA% WE control = 22, WE-Lp = 55, WE-Pp = 9 , WE-Lb_t = 86, WE-Pp_t = 2) and *Listeria* (RA% WE Pp_t = 36) in larvae reared on WE diet. *T. molitor* fed with WB diet presented prevalence of the genera *Lactococcus* (RA% WB control = 41, WB-Lp = 12, WB-Pp = 14, WB-Lb_t = 7, WB-Ppt = 14) and *Staphylococcus* (RA% WB control = 53, WB-Lp = 21, WB-Pp = 29, WB-Lb_t = 26, WB-Pp_t = 1). The family of Enterobacteriaceae is also widely present.

In all the samples, *Pediococcus pentosaceus* was detected from day 0 through-out the assay in all conditions at the species level and *Lb. plantarum* was not detectable at all.

Impact of diets and probiotics on yellow mealworm microbiota

At day 0 the impact of probiotic provision was significantly different for *T. molitor* individuals fed with WB and WE based diets. WE presented alpha-values index Chao1 ($p > 0.05$), Shannon ($p = 0.00056$), Simpson ($p < 0.0001$), InvSimpson ($p = 0.0092$) showing significant differences in species richness of the larvae exposed to the different probiotic treatments. Paired comparisons highlighted differences between control (no) and *Lb. plantarum* deactivated (Shannon: $p = 0.008$; Simpson: $p = 0.001$; Inv.Simpson: $p = 0.016$) and *P. pentosaceus* live and *P. pentosaceus* deactivated (Shannon: $p = 0.0135$; Simpson: $p < 0.0001$). WB presented alpha values index non-significant different in relation to probiotic treatments effects on microbial community composition. These findings suggest that the addition of egg-white protein (WE) influences directly or indirectly on the probiotics and the commensal bacterial composition.

Overall, when the microbial communities were compared over time, after 14 days of probiotic removal, alpha values for OTU composition were not significantly different, suggesting little diversity between the larvae exposed to the different treatments. Meanwhile, variation was found within each treatment over time. Larvae fed with WE presented significant differences in alpha values in pair-wise comparisons between day 0 and day 14 (Chao1: $p = 0.002$: Shannon: $p < 0.0001$; Simpson: $p < 0.001$) and between day 3 and day 14 (Chao1: $p < 0.001$: Shannon: $p = 0.0001$). WB samples presented significant differences in alpha values in pair-wise comparisons between day 0 and day 14 (Chao1: $p < 0.0001$; Shannon: $p < 0.0001$; Simpson $p < 0.001$) and between day 3 and day 14 (Chao1: $p < 0.00001$, Shannon $p < 0.00001$, Simpson $p = 0.023$,). These results are illustrated in the principal coordinate analysis (PCoA) of WB and WE diets (Fig. 6). There is a clear clustering of the data. The day 14 clusters are different from day 0 and day 3 in which more overlap is observed for both WB and WE. Interestingly when comparing the WB and WE, the clusters are differently positioned in the PCoA.

Comparison between live and deactivated probiotic treatments, allowed to define the differences at genus level in the microbial community composition. Larvae fed with live *P. pentosaceus* KVL B19-01 (WB_Pp) presented higher abundances of species in the genera *Enterococcus* and *Sphyngobacterium* in comparison to the ones provided with the deactivated strain (WB_Ppt). The same vital strain inoculated in WE diet resulted in higher abundances of *Enterococcus*, *Acinetobacter*, *Enterobacter*, *Erwinia* and *Pantoea*.

Lactobacillus plantarum WJB had no major impact on the microbial community composition in WE fed larvae in comparison to the control ones. Meanwhile in WB supplemented with the live bacteria, the abundance of species in the genera *Sphingobacterium*, *Chryseobacterium*, *Staphylococcus* were higher, while a lower abundance of *Acinetobacter* was noticed.

Comparison between the probiotic treatments and the day of sampling was made by multivariate ANOVA. The two diets showed different results. A significant interaction effect occurred between probiotic treatment and day on microbial composition for larvae fed on WE diet at day 14 ($R^2 = 0.222$, df = 8, F = 2.66, p = 0.0015). For WB diet this interaction was not significant ($R^2 = 0.08936$, df = 6, F = 1.5873, p = 0.1139).

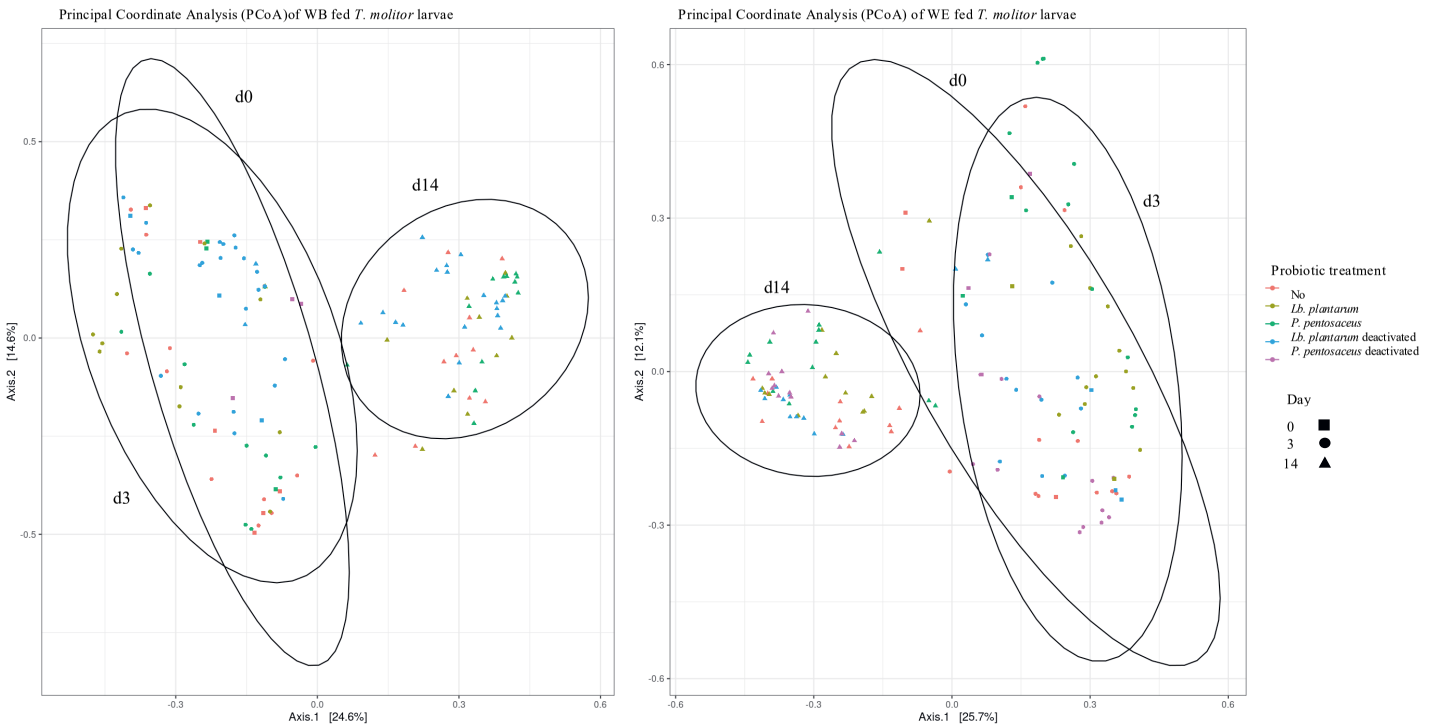


Figure 6. Principal coordinate Analysis (PCoA) of Wheat Bran (WB)(left) and Wheat bran and dried egg white (WE)(right) fed larvae treated with probiotic treatments (control = red, *Lb. plantarum* = yellow, *P. pentosaceus* = green, *Lb. plantarum* deactivated = blue, *P. pentosaceu*s deactivated = pink) until day 0 (square) and then fed with control diet from d3 (circle) and d14 (triangle) samples present a clustering at day 14, showing difference in microbial community composition 14 days after treatment removal and homogeneity of microbial community composition between the treatment groups within each day.

Effects of diet composition on probiotic persistence

Wheat bran (WB) and wheat bran-white egg (WE) based diets did not influence the microbial community composition at day 0 in uninfected larvae (multivariate ANOVA, $p > 0.05$). The genera *Staphylococcus* (relative abundance % RA = 10-50%), *Enterobacteriaceae* (RA=24%), *Chryseobacterium* and *Sphingobacterium* (RA=5-16%) and *Lactococcus* (RA=3-12%), *Pseudomonas* (RA=8%) and *Weisella* (RA=3-7%) being the most represented genera in larvae from all the control treatments. Among diet treatments, a difference in the abundance of the OTUs containing *Pediococcus* genus at day 14 was higher but not significant in WE fed larvae compared to WB fed larvae.

Effects of probiotic provision on microbiota of pathogen infected larvae

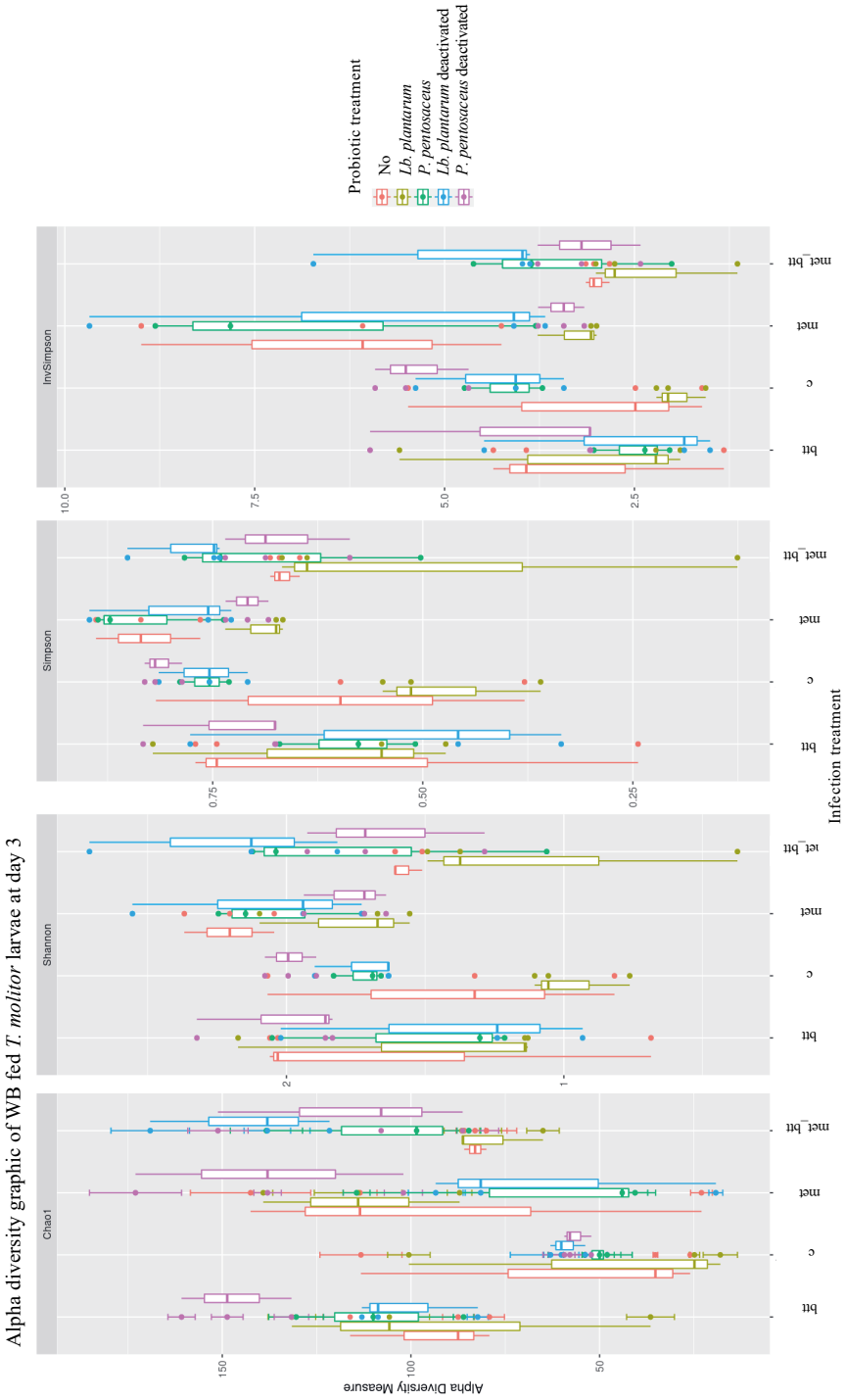
Tenebrio molitor larvae were exposed to pathogens from day 0 to day 3 (72 hours), then placed in sterile cups and provided with non-contaminated feed for 11 days. Overall, the addition of probiotics resulted in significant differences in the larval microbial community composition both at day 3 and at day 14 in the case of infection.

The probiotic treatments supplemented in WB and WE resulted in significant effects on microbial community composition at day 3 ($R^2 = 0.2286$, $F = 5.74$, $df = 4$, $p = 0.0001$; $R^2 = 0.2394$, $F = 6.7579$, $df = 4$, $p = 0.0001$) and at day 14 ($R^2 = 0.1735$, $F = 4.0275$, $df = 4$, $p < 0.00001$; $R^2 = 0.1673$, $F = 4.831$, $df = 4$, $p < 0.00001$). When considering the effect of probiotic species on pathogen-infected larvae in the two diet treatments at day 3, the analysis showed no significant effects in WB fed larvae ($p > 0.05$) but significant differences in WE reared larvae ($R^2 = 0.0739$, $F = 2.9021$, $df = 12$, $p = 0.0001$). Indeed, the microbial community of larvae exposed to single infection of either Btt or Mb or to co-infection presented different microbial community compositions at day 3 in comparison to uninfected individuals in WE (Chao1 control-Btt $P < 0.0001$, control-Btt/Mb $p = 5.468e\text{-}7$, control-Mb $p = 0.00031$) while in WB diet treatments (Chao1 control-Btt $p = 0.00190$, control-Btt/Mb $p= 0.02439$, Shannon control-Mb $p = 0.02544$, InvSimpson control-Mb $p = 0.01369$), indicating that in WB the effect of pathogens on the microbiota composition was less than in WE. (Fig. 7).

After 3 days of *M. brunneum* exposure, even if the fungal pathogen was not detectable by the technique, *Lactococcus* (RA 46%) was the most abundant genus followed by *Weisella*, *Staphylococcus* and Enterobacteriaceae. The controls presented a similar microbial composition with *Chryseobacterium* and *Sphingobacterium* genera have been detected with RA ranging in between 20-53% values in infected larvae at day 14. The individuals reared on probiotic treatments presented lower RA of the same genus (RA<10%). The presence of *Bacillus* genus was of 3% relative abundance in infected individuals at day 3, with lowered values in all probiotic treated larvae (< 2%) and with a complete disappearance at day 14.

Interactions between probiotic treatments and infections are evident at the end of the experiment ($R^2 = 0.3478$, $F = 3.3468$, $df = 12$, $p < 0.0001$; $R^2 = 0.2941$, $F = 2.4474$, $df = 12$, $p < 0.0001$). Indeed, effects of the treatments on OTU abundance are still detectable within control individuals and larvae infected with Btt (Chao1 $P = 3.978E\text{-}06$, Shannon $p = 1.446E\text{-}06$, Simpson $p = 0.002475$, InvSimpson $p < 0.0001$). At day 14, WE fed larvae infected with Btt, Mb or with both pathogens presented significant differences in microbial community composition (Mb-Btt/Mb: Chao1 $P < 0.0001$, Shannon $p = 0.00997$, Mb-Btt Chao1 $p = 0.00814$, Shannon $p = 0.00577$, InvSimpson $p = 0.00094$).

The same trend is not observed in WB fed larvae for which the infection treatment does not show any significant impact on the microbial composition at day 14 ($p > 0.05$). The Bacillus genus was completely absent in samples fed with WE or WB diet supplemented with deactivated probiotic species.


Alpha diversity graphic of WB fed T. molitor larvae at day 3
Probiotic treatment
No
Lb. plantarum
P. pentosaceus
Lb. plantarum deactivated
P. pentosaceus deactivated
Chao1
Shannon
Simpson
InvSimpson
Alpha Diversity Measure
Infection treatment
btt
c
met
met_btt

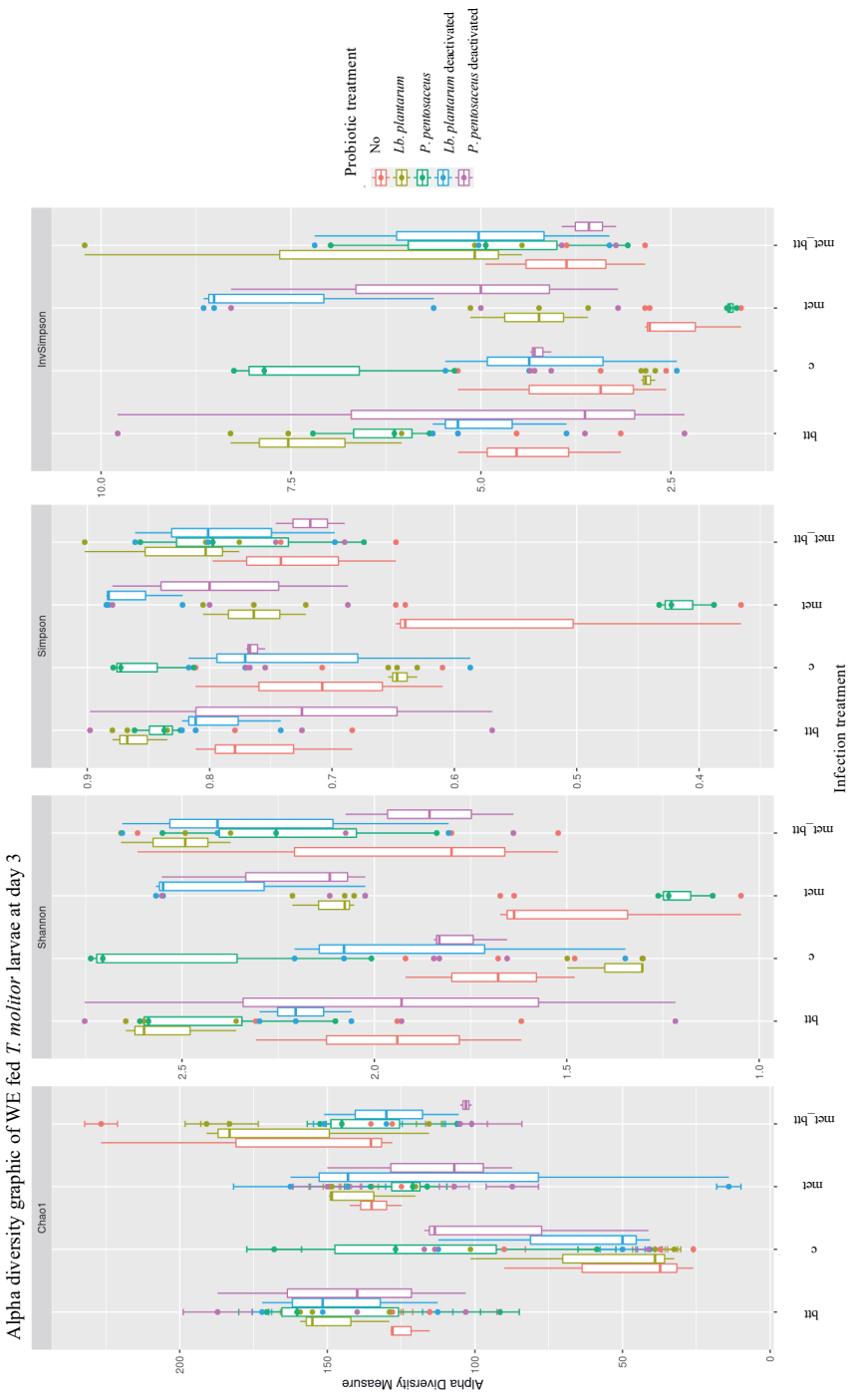


Figure 7. Alpha diversity values of *T. molitor* larvae grouped by probiotic treatments (no = red (control), *Lb. plantarum* = yellow, *P. pentosaceus* = green, *Lb. plantarum* deactivated = blue, *P. pentosaceus* deactivated = pink) and infections (control, btt, met, met_btt) at day 3. The box plot shows the OTU abundances between the grouped samples.

4. Discussion

In this study, yellow mealworm larvae, reared on wheat bran with or without egg white as an additional protein source and with or without two potentially probiotic strains were exposed either to one of two pathogens or a combination of both. The recorded data were larval survival, larval growth and impact on the bacterial microbiota composition. The main purpose of the study was first to investigate whether the addition of probiotics to the feed in the early development stages of *T. molitor* would protect the larvae from harmful impact of pathogens. Secondly to analyse whether a protein enriched diet could similarly increase larval health and performance and lastly to get information about the impact of the treatments on the larval microbiota and the persistence of the probiotics and pathogens.

Following exposure, the larvae showed increased survival in case of co-infection when fed with both live and deactivated *P. pentosaceus* KVL B19-01, and increased developmental rate, expressed as individual mass mean, when fed with *Lactobacillus plantarum* WJB. While the methodology differed in the present study, our results appear in accordance with previous research that found that both live and deactivated *P. pentosaceus* addition increased *T. molitor* larval weight (Lecocq et al., 2021) and live *P. pentosaceus* also increased larval survival following challenge by a pathogen (Dahal et al., 2022). The mechanisms behind the observed results are not yet fully understood, especially in the case of the deactivated *P. pentosaceus* cells. However, other studies have demonstrated that, in some cases, the addition of dead microbes can be as effective as live ones by serving as a source of protein or by acting as immune stimulator (Kataria et al., 2009; Keebaugh et al., 2018).

Pediococcus pentosaceus KVL B19-01 provision resulted in increased survival in case of *B. thuringiensis* infection or *B. thuringiensis* and *M. brunneum* co-infection when supplemented in WB diet. Overall, the WE fed individuals presented a high level of survival >75%, resulting in non-significant effect of probiotic. The effect of dried white egg inclusion in the diet on larval survival suggests a stimulatory effect of this protein source on the insect immune system. The importance of proteins in insect feed in insect development and behaviour has been explored in several species (Riddell and Mallon, 2006). The presence of lysozyme among the proteins of the egg white (Stevens, 1991) may have an antibiotic effect (Ferraboschi et al., 2021).

The relative growth of the larvae was affected only by the presence of the pathogens and not by the probiotic treatments or the diet composition. The presence of both pathogens resulted in the lowest weight gain, followed by the single infection with Btt and Mb. Interactions between pathogens and the insect gut lumen could activate immune and metabolic pathways which cost energy and may result in decreased weight gain (Dolezal et al., 2019) resulting in lower general fitness.

The results of the gut microbial composition of the larvae showed that *Lb. plantarum* provided to the larvae was not detectable in the guts by the end of the experiments and thus did not colonize the insect guts in the long term. This effect is similar to that observed in a study by Storelli et al. (2018) in *Drosophila melanogaster*. On the other hand, *P. pentosaceus* was detectable during the whole experiment in all the samples indicating its natural presence in *T. molitor* microbiota community. *Pediococcus pentosaceus* and *Lb. plantarum*, both live and deactivated forms, have impact on the abundance of the microbiota species of the larvae when present in the feed. After probiotic treatment removal and rearing on the control diets, the bacterial species composition of the microbiota and their abundance was comparable to

previous studies in which the main phyla were represented by Proteobacteria, Firmicutes and Bacteroides (Przemieniecki et al., 2020; Rizou et al., 2022). Microorganism ability to colonize insect gut could be further screened by Whole Genome Shotgun sequencing or using specific primers for determining their abilities prior to application (Lugli et al., 2022). The detection of metabolic active probiotic bacteria can be performed by cDNA sequencing and Oxford Nanopore Technologies (Winand et al., 2019).

The presence of lactic acid bacteria including species of the genera *Lactococcus*, *Lactobacillus* and *Weissella* could increase the presence in the gut of antimicrobial molecules as bacteriocins, organic acids, hydrogen peroxide, acetaldehyde, acetoin, and carbon dioxide (Arena et al., 2018) protecting the yellow mealworm from entomopathogen infection as observed in honey bees and *Galleria mellonella* (Iorizzo et al., 2022; Savio et al., 2022). Interestlingly , larvae exposed to the fungal infection with *M. brunneum* resulted in higher abundance of *Weisella* and *Lactococcus* species suggesting a protective role due to their gut colonisation abilities (Schmidt and Engel, 2021) and production of phenyl-lactic acid and nisin (Arena et al., 2018; Le Lay et al., 2008).

The presence of *Acinetobacter* and *Sphingobacterium* species might be explained by the preference of *T. molitor* for cereal-based diets which digestion requires the action of cellulolytic enzymes provided by the gut microbiome as observed in the European corn borer *Ostrinia nubilalis* and the Colorado potato beetle *Leptinotarsa decemlineata* midguts (Vilanova et al., 2012).

Exploitation of other bacterial species from the genus *Clostridium* and the families *Enterococcaceae* and *Enterobacteriaceae* could take place considering their abundances in the yellow mealworm microbiota and the proven probiotic effect on coleopterans such as *T. molitor* (Rizou et al., 2022) and *Tribolium castaneum* (Grau et al., 2017) and insects from other orders such as *Musca domestica* L. (Diptera: Muscidae)(Zhang et al., 2021). Specific precautions are required prior to approval as probiotic since important pathogens belong to the same families (Al Atya et al., 2015; Gaspar et al., 2009).

Gut microbiota - host interactions are important for increasing digestion and nutrients absorption (Pan et al., 2023). As observed in previous studies (Montalbán et al., 2022), the abundance of the genera varies depending on the composition of the provided diet, reducing the presence of species able to produce cellulolytic enzymes such as *Acinetobacter* spp. in the larvae fed with higher dietary protein content, in our study dried egg white. The diet composition resulted in a difference in the abundance of the OTUs, containing higher loads of *Pediococcus* genera at day 14 in WB fed larvae, compared to wheat bran (WE) diet.

Larval performance may not be the direct causative mechanism of the probiotic acting as nutrient source. Rather, the mechanism could be a direct interaction of *P. pentosaceus* KVL B19-01 with larval immunological or physiological mechanisms, leading to increased growth and immune resistance to pathogens. Further research on insect genetics and metabolomics is needed to elucidate the effects of probiotic provision in the yellow mealworm larvae.

As the study did not take into consideration the whole life cycle of the yellow mealworm, in our case it was not possible to establish the effects of infections and probiotic provision on reproduction rate and life cycle duration as done in previous studies (Lecocq et al., 2021; Dahal et al., 2022).

Moreover, the study of the persistence of pathogens and probiotics in the larvae was possible due to controlled experimental conditions, characterized by timed provision of feed and frass removal (Fig. 2). On the other hand, insect mass-rearing facilities would offer a scenario characterized by lower control levels determining the need to follow good manufactural practices (GMP) for assuring insect and human safety (EFSA, 2015b) and the possibility to monitor microbial species to assess the quality of the rearing system along with probiotic provision (Dupriez et al., 2022b; Savio et al., 2022).

5. Conclusions

The use of by-products as insect feed substrate might increase the risk of entomopathogen infection in *T. molitor* mass-rearing. In this study we demonstrate that by supplementing the feed with vital and deactivated forms of an isolate of the bacterium *P. pentosaceus*, it is possible to reduce the impact of fungal and bacterial pathogens particularly in co-infection conditions. However, the same effect was not observed when another bacterial isolate, *Lb. plantarum*, was provided, indicating specificity in host-symbiont relationship. Moreover, *T. molitor* larvae did not show improved performance after probiotic provision, possibly due to the use of metabolic energy for the activation of immune pathways thereby inhibiting growth. In addition, dynamics of microbiota composition indicate the inability of both the pathogens and beneficial symbionts to persist or increase in abundance in the larvae after their provision ended, pointing to the importance of the microbial community in maintaining insect health.

Author contribution

Conceptualization: C.S., P.H., C.N.-L.; Writing original draft: C.S.; Writing – Review and editing: C.S., C.N.-L., J.J.A.v.L.; Visualization: C.S.; Project administration: C.S., C.N.-L.; Investigation: C.S., A.R.; Data curation: C.S.; Visualization: C.S.; Resources: C.N.-L., J.J.A.v.L., A.B.J.; Funding acquisition: C.N.-L., J.J.A.v.L., A.B.J.

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6

Chapter 6

Effects of feed composition and the probiotic *Pediococcus pentosaceus* on *Tenebrio molitor* growth, protein content and lipid composition

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Abstract

The yellow mealworm (*Tenebrio molitor* L.) is considered one of the most promising candidates among edible insect species as alternative protein source due to its capacity to convert byproducts into high value proteins with a low environmental footprint. The yellow mealworm shows plasticity in nutrient composition in response to the composition of the feed it is provided with. In this study, growth rate, protein content and lipid composition of yellow mealworm larvae were determined after rearing for 50 days on nine diets with either standard 100% wheat bran (WB) medium, or two WB media with lower protein contents through dilution with potato starch, each of these three supplemented with either vital or deactivated *Pediococcus pentosaceus* KVL B19-01 bacteria. Probiotic provision resulted in faster growth in the first 40 days, however, body mass after 50 days of feeding did not differ between the larvae reared on all nine diets. Larval crude protein content was significantly influenced by diet composition with significantly lower content (30.81% DM) in larvae that fed on the diet with the lowest protein content (7.93% DM). Supplementation of vital or deactivated *P. pentosaceus* did not influence larval crude protein or lipid contents. However, larval fatty acid profiles shifted on probiotic-supplemented diets and were characterized by an increase in mono- and poly-unsaturated fatty acids, in particular oleic (C18:1 n-9), linoleic (C18:2 n-6) and arachidonic (C20:4 n-6) acids. The results highlight the possibility to obtain yellow mealworm larvae with a fatty acid profile that is nutritionally more favorable for humans and animals.

1. Introduction

The yellow mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) is an edible insect species considered suitable for mass-rearing (Veldkamp et al., 2012). The low environmental footprint of its large-scale production rearing (Grau et al., 2017) and its capacity to upcycle byproducts from food production to high-nutrient biomass (van Raamsdonk et al., 2017; Veldkamp et al., 2012) are two major advantages in gaining a growing market position (Lou et al., 2021; Montalbán et al., 2022; Rumbos et al., 2021; Thrastardottir et al., 2021). This economic landscape is nowadays incentivating the rising number of companies rearing the yellow mealworm in Europe (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) et al., 2021; Thrastardottir et al., 2021).

The nutrient composition of the yellow mealworm makes the species suitable for food purposes (FAO, 2013) especially due to the high concentration of proteins with an amino acid composition that meets human dietary requirements (Paul et al., 2016; Yi et al., 2013). However, the nutritional content of the insect varies depending on feed quality and environmental conditions (Oonincx and De Boer, 2012). Yellow mealworm fresh body crude protein content average is 19% and is affected by protein content of the diet (Adámková et al., 2020). Lipids are physiologically important constituents with functions in immunity and energy metabolism (Arrese and Soulages, 2010). Yellow mealworm average total lipid content is 32% on dry matter basis (Kim, Seong Hyeon et al., 2012; Paul et al., 2017), presenting higher values compared with Orthoptera (10-15% lipid content), and lower values in comparison with other insect groups such as Lepidoptera (up to 44% lipid content) (Guil-Guerrero et al., 2018). Feed lipid composition influence yellow mealworm lipid concentration and fatty acid composition (Fasel et al., 2017). The fatty acid (FA) profile of lipids is mainly defined by the amount of unsaturated and saturated fatty acids and the ratio of poly-unsaturated (PUFAs) to saturated fatty acids, favorizing the presence of PUFAs and finding in ω-3/ω-6 values that denote a prevalence of ω-3 (Paul et al., 2017). The ability of yellow mealworms to synthetize fatty acids that are essential for mammalian metabolism (Sinclair et al., 2002) increases their value for food application due to the beneficial effects of these essential FAs on human health by reducing the occurrence of coronary heart diseases, depression and behavioural disorders (Fasel et al., 2017).

Probiotic supplementation of feed formulations has been tested for other animals of economic interest (piglets, salmon, trout) demonstrating promising increase in their performance (Duan et al., 2023; Merrifield et al., 2010; Verschuere et al., 2000). Interactions between insects and probiotics also result in several beneficial consequences as has been demostrated for the yellow mealworm and the black soldier fly (Savio et al., 2022). For example, insects' symbionts can play a key role in influencing insect traits such as immune responses and social behaviour (Engel and Moran, 2013). Nutritional benefits of probiotics for insects may be facilitation of digestion in the gut lumen, production of enzymes responsible of feed macromolecule degradation and the production and release of nutrients (Augustinos et al., 2015; Chen et al., 2016; Somerville et al., 2019). Other benefits that have been suggested are the interactions of the microbiota with the gut receptors, activating metabolic pathways that may result in improved insect performance as reduced developmental time and increased growth rate as observed in *Drosophila melanogaster* (Consuegra et al., 2020; Storelli et al., 2018). However, effects of probiotic provision on metabolism and chemical composition yellow mealworm have not been investigated thus far. Insect exposure to the active and inactive probiotic strain could trigger metabolic pathways leading changes in larvae composition as previously observed by Rizou et al. (2022) on *T. molitor* larvae decrement in lipids composition after *Bacillus subtilis*

provision. Moreover, probiotic feed supplementation may be a strategy for mitigating the presence of entomopathogens such as *Bacillus thuringiensis* in insect rearing systems (Savio et al., 2022; Slowik et al., 2023).

In this study we provided *Pediococcus pentosaceus* KVLB 19-01, a strain that has been isolated from the yellow mealworm gut of which probiotic activity has been previously described by Lecocq et al. (2021). It is also known for its probiotic activities in humans and its ability to produce bacteriocines (Todorov et al., 2023). However, there are few studies on the effects of probiotic supplementation on insect development and nutrient composition (Savio et al., 2022).

The aim of this study was to evaluate if the addition of the probiotic bacterium *P. pentosaceus* KVL B19-01, either as living cells or deactivated, to diets differing in protein and carbohydrate contents affect survival, development time, biomass growth rate, as well as body chemical composition of *T. molitor* larvae.

2. Material and methods

2.1. Insect rearing and feed preparation

Tenebrio molitor larvae were obtained from the company Ynsect (Evry, France) and reared at 26°C, 60% relative humidity (RH) and dark environmental conditions at the Laboratory of Entomology (Wageningen University, the Netherlands). The rearing diet of the mealworm was composed of wheat bran. The control diet composition was 100% wheat bran (WB100) and two experimental diets containing a medium and low protein content, obtained by replacing wheat bran by potato starch (Duchefa, The Netherlands): 80% wheat bran-20% potato starch (WB80/PS20) and 60% wheat bran-40% potato starch (WB60/PS40). Additional experimental diets were prepared by adding freeze-dried *P. pentosaceus* KVL B19-01 (Pp) (Department of Plant and Environmental Science, University of Copenhagen, DK) probiotic bacteria or freeze-dried and deactivated (autoclaved at 121°C for 15min) *P. pentosaceus* KVL B19-01 (Ppt) to obtain a concentration of 10^7CFU/mg feed (Lecocq et al., 2021). Table 1 gives an overview of the composition of the experimental diets.

2.2. Experimental design of the feeding trials

For each of the nine diets, in three replicates, twenty females and 20 males of *T. molitor* were combined in plastic containers (volume 1 L; Stackables, UK), giving in total 27 containers. After 5 days the adults were removed and eggs laid and the hatched larvae were kept on the same diet in the same containers during the whole 50 days experiment. The about 300 larvae in each container were offered 40 g of diet, ensuring *ad libitum* feed provision. Water agar 1% (Invitrogen, Thermo Fisher Scientific, France) was provided once a week as water source. The experimental setup was tested in in a climate-controlled room at 26°C, 70% RH in the dark.

Table 1. Composition of the experimental diets.

	Experimental diets								
	WB100	WB100_Pp	WB100_Ppt	WB80/PS20	WB80/PS20_Pp	WB80/PS20_Ppt	WB60/PS40	WB60/PS40_Pp	WB60/PS40_Ppt
Ingredient (%)									
Wheat bran	100	100	100	80	80	80	60	60	60
Potato starch				20	20	20	40	40	40
Probiotic strain added									
P. pentosaceus[a]		x			x			x	
P. pentosaceus[a] inactivated			x			x			x

[a]*Pediococcus pentosaceus* KVL B19-01 isolated from *T. molitor* gut (Lecocq et al., 2021)

2.2.1. Larval growth measurements

Larval growth was measured by recording the mean fresh body weight of a sample of 50 larvae from each batch on five time points, starting on day 18 and ending on day 50. The 50 larvae were then returned to the respective containers.

2.2.2. Sample preparation for chemical analysis

The larvae were sampled when an average individual larval bodymass of *ca.* 100 mg at day 50 was reached. The larval samples were washed with cold water and stored at -80°C. Samples of the diets were also taken before provision to the larvae and stored at -80°C. Larvae and diet were freeze-dried for 48 h at -54°C at 0.100 atm (Alpha 1-4LSCbasic, CHRIST) before being grinded with an electrical grinder (Safecourt Kitchen, NL) for 20 s. Samples were then stored at -80°C.

2.3. Chemical analysis of crude protein and fat contents

2.3.1. Crude protein content

Crude protein content was analyzed for each of three biological replicates of each in two technical replicates. The high temperature combustion DUMAS method was applied for determining the crude protein content of experimental diets and insects. Freeze-dried and grinded samples were weighed with a microbalance (Mettler-Toledo XA105, NL) in duplicate and blocked-in tin capsules for solids (10x10 mm; Inter Science, NL). Cellulose (Aldrich 310697-50 g) was taken as blank control and D-methionine 99% (ACROS Organics227210250) was analyzed as standard reference. The analyses were performed with the Flash EA 1112 Protein analyser (DUMAS) and Eager 200 software instrument. The nitrogen-to-protein conversion factors of 5.26 for the diet (ISO 16634-1:2008) and 4.76 for *T. molitor* larvae (Janssen et al., 2017) were applied to determine crude protein content.

2.3.2. Fat content and methyl esters fatty acids composition

Crude fat content was quantified on samples pooled from the three biological replicates of each diet in two technical duplicate analyses and are reported as the mean of the two duplicates. Crude fat content of diets and larvae was determined by Soxhlet solid-solvent extraction following the official protocol (AOAC Official Method 991.36). The dried and grinded samples (4.4-5.0 g) were packed in a thimble and the fat was extracted with petroleum ether (Merck 1.01774.2500) at 40-60°C for 4 h. When the extraction was completed, the evaporation of the solvent was performed under vacuum conditions with the Vacuum Rotary Evaporator (10 min, 60°C, 600-300 mbar, 70 rpm) (Buchi, NL) (Fig. 1). The samples were derivatized by addition of n-hexane (1/10 v/v) and methanolic KOH (2N, 1/4 v/v), homogenization and centrifugation (5 min, 3500 rpm) to obtain fatty acid methyl esters (FAME). They were then collected in 2 ml glass amber tubes (8 mm, 2 ml, Phenomenex) for performing the qualification and quantification of the fatty acid methyl esters by GC/FID analysis (1:1 v/v in hexane). A Trace Ultra Interscience DSQ II (Thermo Scientific) GC-FID with a 30 m/0.25 mm ID column (Famemax, CAS 12497964820) was used. The standardized F.A.M.E. Mix C8-C24 (Sigma-Aldrich) was diluted to obtain concentrations of 500, 1000, 1500 and 2000 ppm to create a calibration curve that was run in five replicates to be used for qualifying and quantifying FAMEs. The operating conditions for the oven were 2 min at 180°C, raised to 240°C at 10°C/min, and finally held at 240°C for 12 min. The carrier gas was hydrogen at a constant

flow of 1.5 ml/min and the detector was a flame ionization detector (FID) held at 250°C. All chromatographic data was analyzed using the software Xcalibur and Chromeleon. The fatty acid profiles of the experimental diets and larvae were evaluated by comparison with the 2000 ppm standard mixture of FAMEs (C8-C24) used for the GC-FID analysis to obtain semi-quantitative profiles.

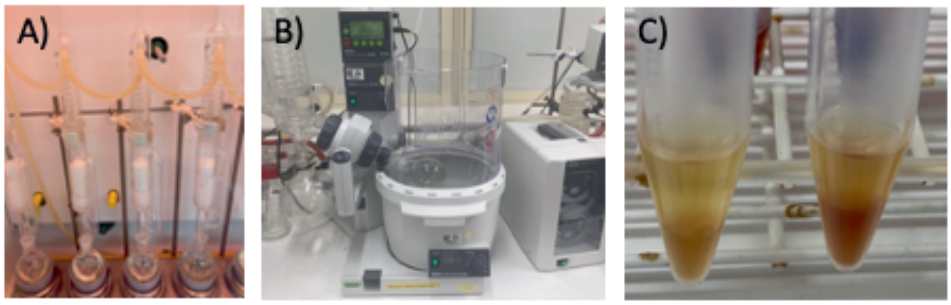

Figure 1. Fatty acid solid-solvent Soxhlet extraction process (A) run for quantifying the crude fat content of diets and larvae; Vacuum Rotary Evaporator (B) used for eliminating the solvent from the samples before the derivatization of fatty acid methyl esters (C).

2.4. Statistical analysis

Statistical analyses were performed using IBM SPSS 24. Growth rate and crude protein content of larvae were analysed using One-way ANOVA to assess the effect of diet, followed by Tukey's honest significant difference (HSD) post-hoc test to compare means. Results are expressed as mean ± standard deviation (SD) applying an alpha value lower than 0.05 as significance threshold.

3. Results

3.1. Larval growth

Larval growth after 50 days of feeding was similar for the nine diets (One-way ANOVA, $F = 1.955$, $df = 8$; $P > 0.05$; Fig. 2). On the diets with the standard protein content (WB100 -16% DM), a tendency was observed toward higher growth on the diet supplemented with deactivated *P. pentosaceus* during the last 10 days. On the diets containing medium (14%DM) and low (12%DM) protein content, growth tended to be faster on the two diets supplemented with either vital or deactivated *P. pentosaceus* than on control diets during the first 40 days. After 50 days of growth larvae on control diets had reached similar body weights.

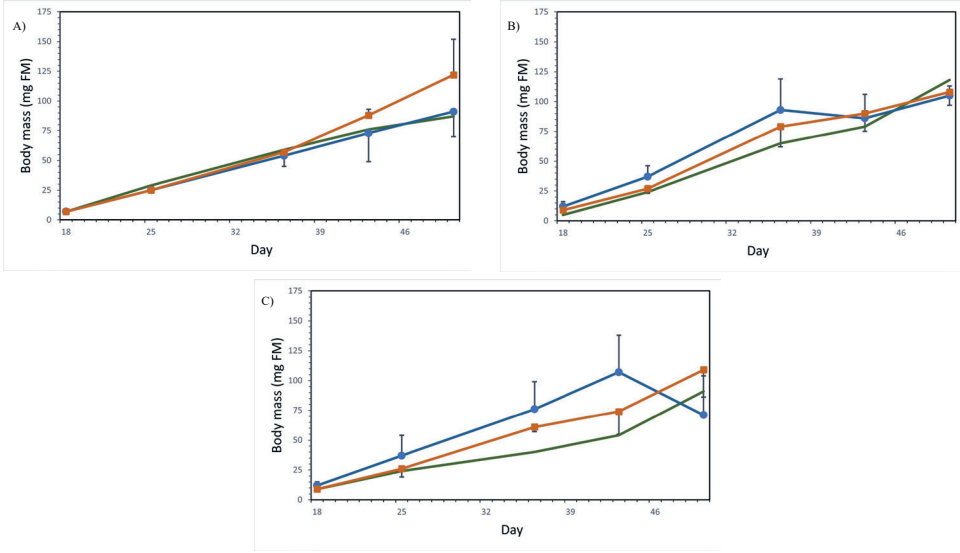

Figure 2. Growth curves of *Tenebrio molitor* larvae feeding on nine diets differing in protein content and supplementation with the probiotic bacterium *Pediococcus pentosaceus*. Mean body weight +/- SD at five time points in the period day 18 – day 50 are shown. A. Growth curves of larvae feeding on WB100 diet (green curve), WB100 supplemented with 10^7CFU/mg feed vital *P. pentosaceus* (blue curve, circles) or WB100 supplemented with inactivated *P. pentosaceus* (red curve, squares). B. Growth curves of larvae feeding on WB80/PS20 diet (green curve), WB80/PS20 supplemented with vital *P. pentosaceus* (blue curve, circles) or WB80/PS20 supplemented with inactivated *P. pentosaceus* (red curve, squares). C. Growth curves of larvae feeding on WB60/PS40 diet (green curve), WB80/PS20 supplemented with vital *P. pentosaceus* (blue curve, circles) or WB80/PS20 supplemented with inactivated *P. pentosaceus* (red curve, squares). To avoid overlapping of line pieces depicting SD values, these values are not shown for the control diets (WB100, WB80/PS20, WB60/PS40); SD values for the other six diets are shown either extending upward or downward from the mean value. All SD values are presented in **Supplementary Table 1**.

3.2. Protein and fat contents of the experimental diets

The crude protein content of differed significantly among the diets (One-way ANOVA; $F = 31.551$, $df = 8$; $P < 0.0001$). The addition of vital or deactivated *P. pentosaceus* KVL B19-01 did not result in significant effects on protein content (Table 2). Crude fat contents of the WB80/PS20 and WB60/PS40 diets that had been diluted with potato starch were lower than of the WB100 diet, as expected (0.82-1.36% DM)(Table 2).

3.3. Chemical composition of *Tenebrio molitor* larvae

Crude protein content (30 -39% DM) of *T. molitor* larvae reared on the nine experimental diets was influenced by diet composition (One-way ANOVA; $F = 3.619$; $df = 8$; $P < 0.002$). Pair-wise comparisons showed that the addition of vital or deactivated *P. pentosaceus* KVL B19-01 had no significant influence on the crude protein content of the larvae (Table 3). Larval crude fat content (4.5 – 15% DM) was quantified with Soxhlet extraction on technical duplicates and results are listed in Table 3.

Table 2. Crude protein and crude fat content of the nine experimental diets (% DM) varying in nutrient composition and presence of vital (Pp) or inactivated (Ppt) *P. pentosaceus* KVL B19-01.

Experimental diet	Crude protein[#] (DM%)		Crude fat[*] (DM%)
	Mean	SD	Mean
WB100	13.56[a]	1.12	1.40
WB100_Pp	14.71[a]	0.70	1.49
WB100_Ppt	13.98[a]	0.49	1.54
WB80/PS20	12.42[b]	1.36	0.87
WB80/PS20_Pp	11.91[b]	0.87	1.08
WB80/PS20_Ppt	11.99[b]	0.93	1.36
WB60/PS40	7.93[c]	0.84	0.82
WB60/PS40_Pp	9.53[c]	1.15	0.97
WB60/PS40_Ppt	8.47[c]	1.57	0.96

Values based on analysis of three replicates, each analyzed in duplicate. *Mean value based on technical duplicates. Means having no superscript letters in common differ significantly. (WB100=100% wheat bran, WB80/PS20= 80% wheat bran 20% potato starch, WB60/PS40 = 60% wheat bran, 40% potato starch)

Table 3. *Tenebrio molitor* larvae crude protein and crude fat content (%DM) reared on experimental diets varying in nutrient composition and presence of vital (Pp) or inactivated (Ppt) *P. pentosaceus* KVL B19-01.

T. molitor larvae	Crude protein[#] (%DM)		Crude fat[*] (%DM)
	Mean	SD	Mean
WB100	39.51[a]	0.035	8.79
WB100_Pp	38.18[a]	0.040	14.22
WB100_Ppt	32.58[a,b]	0.060	12.26
WB80/PS20	33.23[a,b]	0.051	8.34
WB80/PS20_Pp	37.50[a,b]	0.045	9.08
WB80/PS20_Ppt	36.74[a,b]	0.062	5.46
WB60/PS40	30.81[b]	0.029	4.76
WB60/PS40_Pp	33.78 [a,b]	0.028	11.04
WB60/PS40_Ppt	33.27[a,b]	0.034	11.40

Values based on analysis of three replicates, each analyzed in duplicate *mean based on technical duplicates. Means having no superscript letters in common differ significantly. (WB100=100% wheat bran, WB80/PS20= 80% wheat bran 20% potato starch, WB60/PS40 = 60% wheat bran, 40% potato starch)

The fatty acid profiles of the experimental diets and larvae were evaluated by comparison with the standard mixture of FAMEs used for the GC-FID analysis to obtain semi-quantitative profiles. The FAME profiles of the nine experimental diets were similar (Table 4).

The fatty acid composition of the yellow mealworms (Table 4) differed from the profiles in their diets by shifts to higher contents of longer-chain and unsaturated fatty acids. In the larval samples oleic acid (C18:1 n-9) was found at higher concentrations in WB100 and WB60/PS40 diets supplemented with the vital and deactivated *P. pentosaceus* than in the WB100, WB60/PS40 and WB80/WB20 diets. The linoleic acid (C18:2) and arachidonic acid (C20:4) were more prevalent, in particular in the larvae reared on the diets containing deactivated *P. pentosaceus.*

Table 4. Semi-quantitative fatty acid composition of experimental diets and yellow mealworms after 50 days of experimental diet provision.

Fatty acid	Experimental diets																	
	WB100		WB100_Pp		WB100_Ppt		WB80/PS20		WB80/PS20_Pp		WB80/PS20_Ppt		WB60/PS40		WB60/PS40_Pp		WB60/PS40_Ppt	
	diet	Tm	diet	Tm	diet	Tm	diet	Tm	Diet	Tm	diet	Tm	Diet	Tm	diet	Tm	diet	Tm
Octanoic acid (C8:0)	=	-	=	-	=	-	=	-	=	-	=	-	=	-	=	-	=	-
Capric acid (C10:0)	=	-	=	-	=	-	=	-	=	-	=	-	=	=	=	-	=	-
Lauric acid (C12:0)	=	-	=	+	=	-	=	-	=	-	=	-	=	+	=	-	=	++
Myristic acid (C14:0)	=	=	=	++	=	+	=	-	=	=	=	=	=	+	=	+	=	++
Palmitic acid (C16:0)	+	++	+	++	+	+	+	=	+	=	+	=	+	=	+	+	+	++
Palmitoleic acid (C16:1 n-7)	=	+	=	++	=	=	=	=	=	=	=	+	=	=	=	+	=	++
Stearic acid (C18:0)	=	-	=	++	=	+	=	+	=	=	=	=	=	+	=	+	=	++
Oleic acid (C18:1 n-9)	+	+	+	++	+	++	+	+	+	+	+	=	+	+	+	++	+	++
Linoleic acid (C18:2 n-6)	+	+	+	+	+	++	+	+	+	+	+	++	+	=	+	++	+	++
Arachidonic acid (C20:4 n-6)	+	+	+	+	+	+	+	+	+	=	+	++	+	+	+	-	+	++
Linolenic acid (C18:3 n-3)	+	+	+	+	+	+	+	++	+	=	+	=	+	+	+	-	+	+
Erucic acid (C22:1)	-	-	-	-	-	-	-	=	-	=	-	-	-	-	-	-	-	-
Docosanoic acid (C22:0)	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Lignoceric acid (C24:0)	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-

- ,= , +, ++ indicate the abundance of FAMEs based on semi-quantitative evaluation of the GC-FID chromatograms in comparison to the FAME standard mixture, C8-C24, each at a concentration of 2000 ppm. (diet: experimental diet; Tm: yellow mealworms)(WB100:100% wheat bran, WB80/PS20: 80% wheat bran, 20% potato starch, WB60/PS40: 60% wheat bran, 40% potato starch ; Pp: vital *P. pentosaceus*, Ppt: deactivated *P. pentosaceus*)

4. Discussion

Feed supplementation with *P. pentosaceus* KVL B19-01 did not result in significant effects on crude protein or lipid contents. Also, the variable composition of the experimental diets did not affect significantly larval growth as measured at the end of the experimental duration, although growth tended to be faster on the low protein content diets supplemented with either the live or deactivated probiotic *P. pentosaceus* KVL B19-01. It opens questions related to the interaction between insect gut receptors and the bacterial cell envelope that could activate metabolic pathways as previously described by Storelli et al. (2011) in *Drosophila melanogaster* fed with the mutualistic symbiont *Lactobacillus plantarum* WJB. Stimulation of insect development is explained by TOR kinase activity that is stimulated in the fat body and the prothoracic gland by which growth rate is promoted by systemic InR signaling and the length of the growth phase is reduced during the larval stage by Ecd production (Storelli et al., 2011). We hypothesize that similar interactions could occur in the *T. molitor* gut environment when fed with beneficial symbionts. Supplementation of feed with the bacterial probiotics *Bacillus subtilis*, *Bacillus toyonensis* or *Enterococcus faecalis* led to increased growth rate of *T. molitor* larvae (Rizou et al., 2022) while in this study the larval growth after *P. pentosaceus* provision in WB60/PS40 and WB80/PS20 diets was characterized by faster weight gain in the first 40 days, however, body mass after 50 days was similar among the nine diets tested. These results are in line with previous studies denoting the ability of the bacterial isolate to increase larval growth rate (Dahal et al., 2022; Lecocq et al., 2021).

The diet has been supplemented with the vital or deactivated *P. pentosaceus* for observing if the vitality of the probiotic bacteria is needed for observing effect on insect performance or variation in nutrient composition. In both cases the provision of the probiotic strain presented improved effects on larval growth in the first days of growth, as observed by Lecocq et al. (2021). In our case, we recorded no differences in between the vital and deactivated bacterial strain while Lecocq et al. (2021) highlighted significative differences after 8 weeks of growth in favor of the vital source. The addition of the probiotic strain in the feed resulted in non-significative differences in terms of fat and protein diets nutritional composition. Insect performances and insect nutrient composition could be then determined by the activation of immune and metabolic pathways due to the interaction with cellular elicitors still apported by the probiotic sources after the inactivation process, as thermostable proteins and cellular membrane glucosides (Koropatnick et al., 2004; Papagianni and Anastasiadou, 2009). Applying deactivated probiotic bacteria (postbiotics)(Salminen et al., 2021) as feed supplements might lead to improved insect performances and reduction of risk of pathogens infections as observed also for mass reared shrimps (Ballantyne et al., 2023). Postbiotics provision could also facilitating the handling and storage of the supplements, reducing concerning related to the total microbial viability at the time of provision (Weese, 2003).

Considering the yellow mealworm nutrient composition, lower values of crude protein (30 - 39% DM) and fat content (4.5 – 15% DM) were observed compared with previous studies on *T. molitor* reared on by-products (45 - 49% DM and 19-28% DM, respectively; Van Broekhoven et al., 2015; 44 - 53% DM and 19 - 36% DM, respectively; Oonincx et al., 2015). We propose that these differences result from the differences in diet composition among the the two studies cited and this study. Van Broekhoven et al. (2015) and Oonincx et al. (2015) formulated diets based on a variety of by-products of food production whereas in this study plain wheat bran and potato starch have been used. The crude fat content of the larvae is in line with the findings of Yi et al. (2013).

The protein content of the yellow mealworm larvae was affected by the diet composition as previously observed Rumbos et al. (2020) and not by the probiotic provision. Analysis of the amino acid profile and on larval digestible proteins would be of interest for determining variations in protein amino acid profile (Yi et al., 2013).

The supplementation with either the vital or the deactivated probiotic tended to increase the crude fat content of the larvae on the WB100 and the WB60/PS40 diets whereas this was not observed on the WB80/PS20 diet. It has been recently demonstrated, that while converting feed varying in lipid profile, the yellow mealworm shows plasticity in the ω-3 PUFA profile in relation to the variation of ω-3/ω-6 feed composition (Fasel et al., 2017). In this study, the provided feed did not vary in fatty acid profile. Higher concentrations of oleic acid (C18:1) were observed in the larvae reared on the WB100 and WB60/PS40 diets containing vital and deactivated *P. pentosaceus* and of linoleic acid (C18:2) and arachidonic acid (C20:4) in larvae reared on WB60/PS40 and WB80/PS20 diets containing the deactivated probiotic. The synthesis of linolenic acid (C18:3) did not change after probiotic provision. These findings point to the possibility to shape the FA profile by bacterial probiotic provision in addition to provision of diets differing in nutrient and fatty acid composition as previously demonstrated by Dreassi et al. (2017), Fasel et al. (2017) and Kröncke et al. (2023). In this study, *P. pentosaceus* in its deactivated form promoted the overall production of PUFAs in the yellow mealworm larvae, suggesting their direct action on metabolic pathways or on the microbial community in the gut lumen (Kataria et al., 2009).

Application of metagenomic techniques for studying yellow mealworm metabolic pathways activation could provide an explanation on variations of insect performances and lipid content after beneficial symbionts provision such as *P. pentosaceus*. Moreover, the possibility to increase PUFA content of the insect body creates opportunities for applications of mealworms for feed and food purposes considering the importance of ω-3 diet inclusion in preserving human health (Bradbury, 2011; Freeman, 2000; Mischoulon and Fava, 2000; Jørgensen et al., 2001).

5. Conclusion

The use of by-products as insect feed substrate might affect insect performance and nutritional value of mass-reared *T. molitor*. In this study we demonstrate that the diet composition and the presence of the probiotics did not strongly affect insect weight gain. The macro-nutrient composition of the yellow mealworm varies in relation to the feed formulation, resulting in lower crude protein content in larvae reared on feed with lower protein content achieved through dilution with potato starch. Moreover, by supplementing low nutrient diets with the deactivated probiotic bacterium *P. pentosaceus*, an increment in MUFA and PUFA concentrations in yellow mealworm larvae were observed, highlighting the potential of probiotic provision for shaping the larval nutritional fatty acid profile for food and feed purposes.

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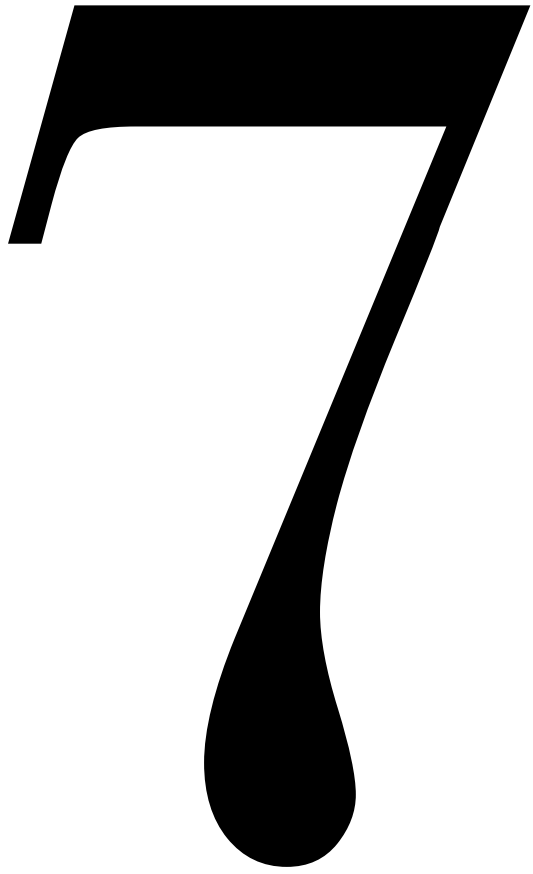

Chapter 7

General discussion

1. Introduction: Sustainable yellow mealworm production for feed and food

Insects have been proposed as sustainable protein source along with plant-based products (Henchion et al., 2017) due to their environmental, economic and social sustainability for assuring food security to the estimated 7 million people who suffered of malnutrition and hunger in 2022 and to the projected increasing global human population to 10 billion in 2050 (FAO et al., 2023).

When comparing insects to conventional livestock, lower land use and water resources have been recorded and GHG emissions are lowered due to the lack of CH_4 emission by most edible insect species (Oonincx and de Boer, 2012; Thornton et al., 2023). Moreover, the efficient use of feed is a parameter to take into account, indeed the yellow mealworm feed conversion efficiency values are comparable to poultry and two to five times higher than pigs and cattle (Van Broekhoven et al., 2015; van Huis, 2013). In addition, almost the whole animal is edible, in comparison to the 40-55% of poultry, pigs and cattle (van Huis, 2013). Meanwhile as mealworms are poikilothermic, the regulation of rearing system temperatures through heating and cooling systems is crucial for assuring a proper development at every developmental stage (Oonincx and De Boer, 2012) which makes energy use in insect mass-rearing similar to other animal food production systems.

The design of production site and business plan takes into consideration the main purpose of the rearing facility (Thrastardottir et al., 2021) and all the drivers that influence the success of the production such as the accessibility to feed supplies, employment conditions, economics, environmental and health hazards are taken into consideration for maximizing consumers' satisfaction (Leppla, 2023). Insect rearing may show up to be a valuable source of animal feed considering the relatively low environmental footprint and nutritional benefits for several animals such as poultry and fish. Meanwhile, scaling up the facilities is needed for increasing production competitiveness in comparison to other feed sources as fish flour and soy meal (Gasco et al., 2023; Niyonsaba et al., 2021). The monetary constraints could be justified by the nutritional benefits of the bioactive compounds they contain such as antimicrobials, medium chain fatty acids, chitin and its derivatives (Borrelli et al., 2021).

Data from International programs such as Insects for Peace show that insect rearing provides a solution for food security also at small production scales, excluding feed import and increasing employment thereby creating an inclusive system (Barragán-Fonseca et al., 2020).

The global Insects as Food and Feed (IAFF) industry is currently producing trillions of insects with a trend of increasing production expected in the upcoming years (Alliedmarketresearch, 2021) for fulfilling the Common Agriculture Policy (CAP 2021-2027) goals along with the creation of new job positions (Halloran et al., 2018; Macombe et al., 2019; Mancuso et al., 2019).

This scenario is increasing the need to settle legislation related to insect welfare for assuring a proper treatment during the stages of production, optimizing the rearing conditions in relation to their biology (Halloran et al., 2018). Several studies are focused on insects' sentience as the production facilities are characterized by a high density environment and a long production process including slaughtering, as for other livestock productions (Fig. 1)(Barrett and Fischer, 2023; Ojha et al., 2021).

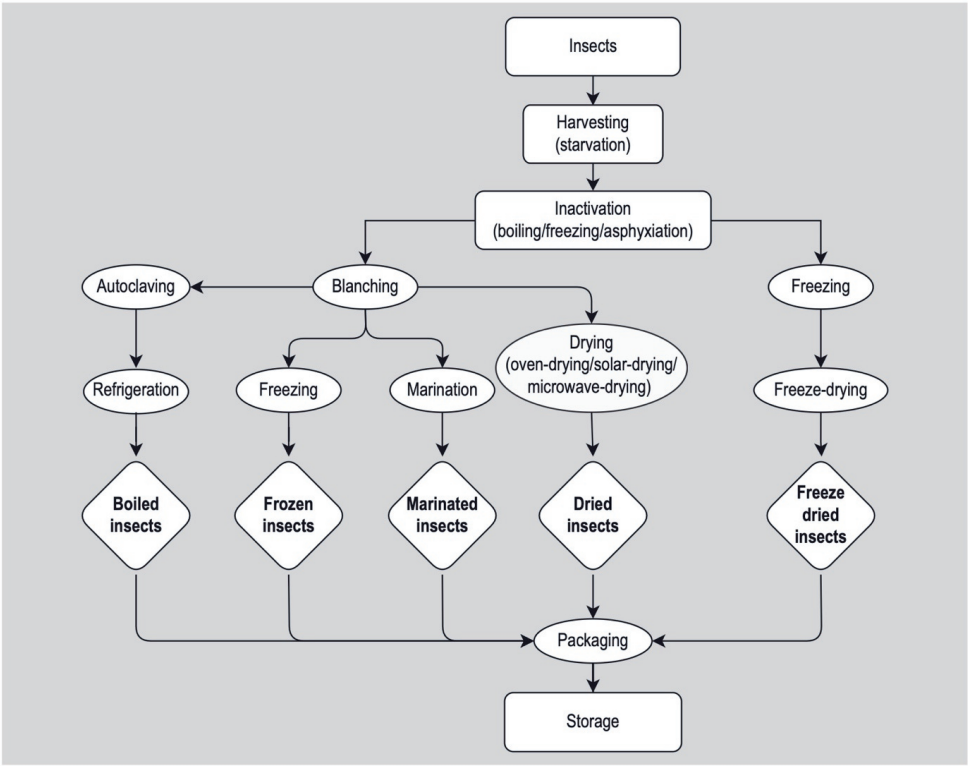

Figure 1. Processing phases of whole edible insects (Ojha et al., 2021).

It might be a possibility to assure insect welfare in mass-rearing facilities through probiotic supplementation and feed formulation (**Chapter 5 and 6**), while taking into consideration the differences between insect species behavior and nutritional need and the rearing environment.

2. Mass-Rearing Environment

The environment of insect mass-rearing can widely differ depending on the dimensions of the production facility, depending on the social context (Berggren et al., 2018). Finding optimal environmental conditions for preserving insect health and maintaining high levels of production quality and quantity are essential for assuring system sustainability (Wade and Hoelle, 2020).

Temperature is a key factor affecting metabolism, growth rate, growth efficiency and macronutrient composition (Bjørge et al., 2018). Survival, developmental rate and fecundity are also highly affected by this environmental parameter, impacting strongly production time, efficiency and quality (Lehtovaara et al., 2018). Optimal growth efficiency of the yellow mealworm occurs at 31 °C (Bjørge et al., 2018). Maintaining the optimal temperature in the rearing system is one of the most costly rearing requirements and solutions have been proposed for cooling the air in the rearing systems to obtain the best performance of the insect (Cortes Ortiz et al., 2016). Moreover, temperature can affect insect-parasite interactions, therefore emphasizing its importance may assure the quality and safety of the rearing by reducing the parasite impact (Herren et al., 2023).

Mechanical stimulation as sieving processing to separate larvae or pupae of different size could determine different larval susceptibilities to opportunistic pathogens or create physical damages to the insect, arresting the development or determining the occurrence of malformations (Dupriez et al., 2022). The rearing systems are characterized by high density levels playing a role in insect development and symbiont transmission (Cohen, 2018; Dupriez et al., 2022; Maciel-Vergara et al., 2021).

3. Assuring insects and consumer safety trough Good Manufactural Practices

Insect mass-rearing requires specific conditions for assuring safety and quality of the production. GMP and HACCP systems as used in conventional food and feed production facilities as optimal guidelines for preserving insects and consumers health are recommended (International Platform of Insects for Food and Feed, 2022). Quality control programs are in place for insects reared for other purposes as in biocontrol programs such as parasitoids and predators widely produced in Europe or for controlling the spread of parasites as the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) reared for applying sterile male technology (Leppla, 2023).

Moreover, avoiding insect pathogens of human pathogens vectored by insects are key measures to assure insects' safety and quality in the rearing system (EFSA, 2015; Ojha et al., 2021). Also maintaining optimal environmental conditions (Herren et al., 2023) and formulating the feed with respect to a given insect's nutritional requirements are important. (Lipke and Fraenkel, 1956).

3.1. Microbial contamination and detection methods

Nowadays substrates from different proveniences are taken into consideration for insect rearing (Oonincx et al., 2015; (Rho and Lee, 2022). The sustainability of the production drives the companies to obtain feed sources easily purchasable as by- or side-products or wastes originating from production sites as close as possible to the rearing setup (Gasco et al., 2020; Montalbán et al., 2022; Pinotti and Ottoboni, 2021). The presence of pathogens or toxic compounds could be due to processing, handling or non-optimal storage and may therefore strongly depend on the origin and nature of the substrates. Several studies are focused on questions related to 'insects' microbial and chemical safety and pathogen transmission, especially in the case where the feed supply comes from organic waste in a circular economy assets (Gasco et al., 2020). Among the mass-reared insects, the yellow mealworm shows the capacity to uptake heavy metals but at concentrations below the safety limits, even after processing (Truzzi et al., 2019; Vijver et al., 2003).

Along with chemical risks, pathogen monitoring in food and feed or environment and their persistence in insects have been studied for safety reasons as it is well known that insects can be vectors of food-borne pathogens such as *Salmonella* spp., *Clostridium* spp., *Streptococcus* spp., *Listeria monocytogenes* and *Vibrio* spp. (EFSA, 2015; Gałęcki et al., 2023). Studies on pathogen transmission show the ability of several mass-reared insects as *Hermetia illucens* and *T. molitor* to maintain a certain level of pathogens in the feed or larva. Indeed, in laboratory studies the persistence of *Salmonella* spp. and *Serratia marcescens* in *T. molitor* larvae and frass were demonstrated (Dupriez et al., 2022; Wynants et al., 2019). In **Chapter 4**, *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* was detected in larvae infected by free-feeding. The pathogens' ability to persist in the larvae highlights the need to assess limit of detection and to follow GMP for assuring safety in insects reared for feed and food (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) et al., 2021).

Methods of pathogen detection for the yellow mealworm have been discussed in **Chapter 3** starting by the observation of infection symptoms, that are often characterized by changes in behavior, color, texture and odor (Slowik et al., 2023). Examples as flaccid consistency and change in color in case of bacterial proliferation are frequently observed (Maciel-Vergara et al., 2021).

Detection methods may apply Artificial Intelligence that combines morphology and color information for predicting the stage of the insect by optical observation (Wu et al., 2019), with infrared technologies (NIR) (Dowell et al., 2005) in order to identify, count, and separate unhealthy individuals from the healthy ones in the production system for preventing and avoiding the spread of diseases. The detection method allows also the separation of individuals by length and number of segments (Majewski et al., 2022). Techniques as FISH (fluorescence *in situ* hybridisation) (Huang et al., 2023), 3D fluorescent microscopy after tissue transparization (Fig. 2 A)(Gualda, 2022; Moya-Andérico et al., 2021), and histological staining (Fig. 2 B)(Polenogova et al., 2021) might be applied for recognizing the pathogens and for observing the location of the infections for further investigations of host-parasite interactions.

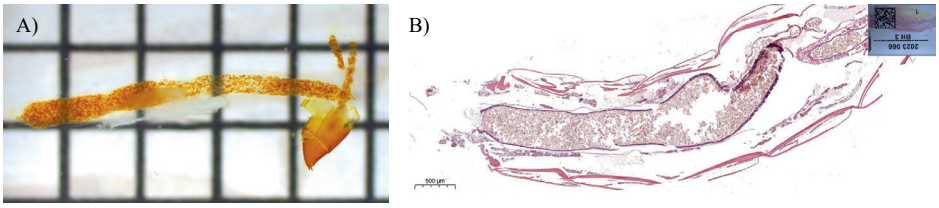

Figure 2. A) *T. molitor* intestine transparization preparation for performing 3D fluorescent microscopy analysis. B) Histological staining of longitudinal 6 µm section of paraffin embedded whole yellow mealworm larvae for histological observations (Preliminary studies at INRAE, Jouy en Josas in collaboration with ABRIDGE and Emerg'in-IERP platforms).

Along with optical techniques, the use of molecular techniques such as the polymerase chain reaction (PCR) is suggested for identifying viral covert infection, the presence of protists (Bass and del Campo, 2020) and bacterial pathogens to the species level (Verma *et al.*, 2017). For species identification the application of molecular techniques such as Next Generation Sequencing (NGS) has become widely available. The use of metagenomics has already revealed novel pathogens in several commonly reared insect species, such as the presence of a new iflavirus in *Acheta domesticus* colonies (de Miranda et al., 2021), highlighting the importance of reference genome databases of different parasites / pathogens for non targeted metagenomic screening approaches (de Miranda et al., 2021). Some entomopathogens with low virulence such as *Serratia marcescens* could be used as indicator species to monitor the hygienic quality level of the rearing system (Dupriez et al., 2022).

3.2 Insect Nutrition and Quality

The geometrical model proposed by Simpson and Raubenheimer (1995) offers an efficient approach for investigating feeding and nutrition of animals, allowing the investigation of nutrient composition levels and availability in diet formulation for maximizing performance (Rho and Lee, 2022).

Minimal nutrient requirements have been widely studied for several insect species of economic interest, identifying the vitamin B group and carnitine as essential components of the diet of *T. molitor* for achieving its development and avoiding adult malformation (Lipke and Fraenkel, 1956).

The safety of the provided feed plays a key role in preserving insect health where the presence of entomopathogens could affect performance in mass-rearing facilities (Maciel-Vergara et al., 2021). In this thesis, the virulence of the bacterial entomopathogens *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* has been studied in *T. molitor* larvae in densities similar to those found in mass-rearing environments. The ability of a given pathogen to persist in the *T. molitor* larvae (**Chapter 4**) indicated the strain could persist in alive larvae. This was not observed in studies for other pathogens such as *Salmonella* spp. and *Campylobacter jejuni* (Strother et al., 2005; Wynants et al., 2019) but *Serratia* was recorded in the larval frass (Dupriez et al., 2022). Further questions related to larval microbial community composition and *Bacillus thuringiensis* persistence were then been explored in **Chapter 5**, where our results demonstrated that the pathogen is not able to persist in the infected larvae kept in low densities.

Insect diet formulation is a critical factor in obtaining insects with an optimal nutrient profile for feed and food consumption (Oonincx and de Boer, 2012; van Huis et al., 2021). Lipids and protein content vary according to the carbohydrate/ protein ratio and to the quality of the lipids present in the feed (Fasel et al., 2017). Metabolism differs between insect life stages. It is possible to model the protein and lipid storage (Oonincx et al., 2015; Van Broekhoven et al., 2015) to meet production needs targeting different sectors like feed, food (Food and Agriculture Organization of the United Nations, 2013; Veldkamp et al., 2012) or cosmetic applications (Franco et al., 2021).

Results from several research papers have proposed the possibility to use the diet as tool for shaping the insect body composition for storing components for specific purposes as for example increasing mineral content for animal nutrition (Oonincx et al., 2015; Van Broekhoven et al., 2015 ; Finke, 2003; Finke and Oonincx, 2014; Klasing et al., 2000; Susan, 2000). It can be achieved by gut loading by providing a specific component for a short period before the insects are harvested (Finke, 2003; Finke and Oonincx, 2014 ; Klasing et al., 2000; Susan, 2000) or by modifying the insect body by providing a specific diet for a long term (Oonincx et al., 2015; Van Broekhoven et al., 2015).

In this thesis, questions related to effects of feed protein content on larval weight gain, microbial community, pathogen susceptibility and larval chemical composition have been addressed by designing two experiments. Wheat bran was used as control diet or mixed with dried white egg (**Chapter 5**) or potato starch (**Chapter 6**) were included to achieve variation in protein content. The diet formulations did not influence larval survival (**Chapter 5**) whereas differences in growth were observed in terms of faster growth in the first 40 days, ending with no significative difference after the following 10 days (**Chapter 6**). The increased protein content of the diet containing dried egg white resulted in non-significant effects on insect weight gain but decreased susceptibility to entomopathogens infection (**Chapter 5**). Reasons behind these findings might be related to the nature of the protein source, for example the presence and content of lysozyme that is known for its antimicrobial activity (Ferraboschi et al., 2021). The presence of the vital and deactivated *P. pentosaceus* improved larval growth on the low protein content diets.

In previous studies, effects on diet composition on mass-reared insects for feed and food purposes were investigated after the provision of diets based on by-products and several carbohydrate/protein contents (Oonincx et al., 2015; Rumbos et al., 2020). As *T. molitor* larvae presented lowered feed digestibility if the diet contains protein contents above 30% (Morales-Ramos et al., 2013), in this thesis the protein content of the diets (**Chapter 5** and **6**) was maintained below 20%.

Other studies reported that higher lipid inclusion in the feed resulted in lower fat concentrations in the larval body and decreased reproduction and performance (Morales-Ramos et al., 2013). In this thesis,

the provided experimental diets did not differ in fatty acid profile and only in larvae fed with the probiotic source increased fat contents were observed in line with what was reported for *Hermetia illucens* and yellow mealworm larvae fed with yeast (Richard et al., 2019). Rizou et al. (2022) observed lower larval fat content after *Bacillus subtilis* or *Bacillus toyonensis* provision and improved larval growth.

Moreover, the semi-quantitative analysis on *T. molitor* larvae fatty acid composition after *P. pentosaceus* provision showed an increase in fatty acid concentration but a lower proportion of saturated fatty acids and favouring mono- and poly-unsaturated fatty acids (**Chapter 6**). This might indicate a correlation between lipid biosynthesis and interactions between host gut receptors and bacterial cell surface molecules like peptidoglycan (PGN), or flagellin (Koropatnick et al., 2004; Nyholm and Graf, 2012).

4. Beneficial symbionts and insect health

Symbiosis refers to a prolonged association between two or more organisms of different species that may last for a lifetime. These relationships can be mutualistic, commensalistic, or parasitic/pathogenic, often not fitting in any of these categories due the complexity of the interactions. A dynamic movement in between antagonism and cooperation can be observed depending on the situations (Fig. 3).

Figure 3. Description of symbiotic relationships that can range from antagonistic to cooperative. The complete understanding of the symbiotic relationship is challenging. Cooperative relationships occur between mutualists, antagonistic interactions between hosts and parasites or pathogens and mixed interactions between commensals.

Harmful and beneficial symbionts can be acquired from the diet and from the environment and the transmission can be horizontal or vertical (Paniagua Voirol et al., 2018; Savio et al., 2022; Slowik et al., 2023). In the order Coleoptera, cases of iflavirus transmission have been studied in *Tribolium castaneum,* indicating the transfer to the offspring and to individuals of the same generation through contaminated frass (Fatehi et al., 2023). Similar approaches were applied to analyse the transmission and persistence of food-borne pathogens such as *Campylobacter jejuni* and *Salmonella* spp. and facultative entomopathogens such as *Serratia marcescens* in *Tenebrio molitor* and *Alphitobius diaperinus* (Dupriez et al., 2022; Wynants et al., 2019; Strother et al., 2005). In **Chapter 4** *in vivo* studies on *T. molitor* larvae from different larval stages demonstrated how the entomopathogen *B. thuringengis* sv. *morrisoni* biovar *tenebrionis* inoculated by free-feeding can persist in the larvae even 11 days after pathogen removal from the substrate, despite the frass having been systematically removed from the environment, which should reduce the probability of horizontal transmission. The results suggested the ability of the pathogen to persist and to establish in the larvae. In **Chapter 5**, 16SDNA sequencing on the larval microbial community 14 days after Btt exposure, showed the inability of the entomopathogen to persist in the larvae. In many publications, a key role are assigned

to the insect-associated microbiota community in determining the colonization and protection against pathogens (Schmidt and Engel, 2021; Rizou et al., 2022).

Studies on vertical transmission of beneficial symbionts showed the protection from parasites and pathogens in the offspring of aphids (Oliver et al., 2005). In the yellow mealworm, cases of vertical transmission have still to be identified. In **Chapter 5**, the protective role of the beneficial symbionts has been studied by providing *Lb. plantarum* WJB and *P. pentosaceus* to young *T. molitor* larvae. Protective effects of *P. pentosaceus* were observed in case of *B. thuringiensis* and *M. brunneum* co-infection. The symbioses can also result in microbial provision of essential nutrients such as amino acids or B vitamins (Blewett, 1944 ; Michalkova et al., 2014; Salem et al., 2014) in order to supply important nutrients in deficient diets such as some by-product or wastes (Pinotti and Ottoboni, 2021). Nutritional symbiosis was observed in coleopteran pests, in which symbiotic bacteria improved cuticular resistance during the molting phase, reducing the risks of fungal infection (Kanyile et al., 2022). Microbial symbionts may be also involved in the degradation of polymers (Salem et al., 2017) or detoxification of phytotoxins and pesticides (Itoh et al., 2018). This ability allows insects to occupy ecological niches that would otherwise be inaccessible. Bacterial strains with bioeconomic application could be isolated from insects exemplified by *Mixta tenebrionis* sp. nov., a strain able to degrade polymers, from *T. molitor* gut (Brandon et al., 2018; Xia et al., 2020; Yang et al., 2018). Moreover, the presence of beneficial microbes can lead to improved larval growth and modification in the nutritional content of *T. molitor* larvae as observed by Rizou et al. (2022) in case of diet supplemented with *Bacillus subtilis* and *Enterococcus faecalis* and in this thesis after provision of *P. pentosaceus* (**Chapter 6**), giving advantages in a mass-rearing context (Savio et al., 2022).

5. Probiotic applications in mass-rearing environments

Probiotics have been recognized as feed supplements in livestock nutrition for several purposes covering health improvement and well-being (Fig. 4)(Zommiti and Ferchichi, 2021). In case of stressful situations and intestinal microbial imbalances, the provision of probiotics can result in the improvement of gut health, assuring nutrient absorption resulting in optimal development and reducing the risk of diseases (Zamojska et al., 2021; Zommiti and Ferchichi, 2021).

Probiotics are defined as "*live microorganisms which, when administered in adequate amounts, confer a beneficial effect on host health*" (Hill et al., 2014), and have to follow specific requirements:

- non-pathogenic;
- adhesion to the epithelial cells;
- colonisation and reproduction abilities in the host;
- ability to survive the passage through the harsh gastrointestinal tract (GIT) conditions;
- high resistance to gastric acidity and bile content;
- produces metabolites to inhibit or kill pathogenic bacteria;
- has undergone trials *in vitro* and *in vivo* that demonstrate its benefits.

The probiotics would have therefore a role in balancing the gut microflora through increasing nutrient absorption, resistance to pathogenic agents, both through a strengthening of the intestinal barrier and stimulating directly the immune system (Hill et al., 2014; Syngai et al., 2016).

The microorganism should remain viable under processing, production, and storage conditions. In **Chapter 2** (Savio et al., 2022), an approach for potential probiotic isolation and screening has been proposed for mass-reared insects. All the steps take into consideration the previously mentioned requirements for assuring the safety of the application. Differences can be observed from applications in other animal rearing systems as for example in poultry where the effects of probiotic provision are

measured using histopathological analysis and sensory and microbiological meat quality (Kabir, 2009). The experiments performed in this thesis followed the scheme proposed in **Chapter 2** for probiotic selection and characterization, determining the *in vivo* provision of the potential probiotic strains only after *in vitro* screening. The strains were selected based on their origin and their known ability to produce antimicrobial compounds such as pediocins and organic acids (Papagianni and Anastasiadou, 2009; Seddik et al., 2017). *Pediococcus pentosaceus* was isolated by Lecocq et al., (2021) from *T. molitor* gut and *Lb. plantarum* WJB was selected due to its previously tested effects on *Drosophila melanogaster* development (Storelli et al., 2011). Both *Lb. plantarum* and *P. pentosaceus* showed *in vitro* antimicrobial activity against the entomopathogens selected for the study, *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* and *M. brunneum* KVL 12-30.

In **Chapter 5**, the non-pathogenicity of both strains was assessed, resulting in no significant mortality in the yellow mealworm. The ability of the potential probiotics to persist in *T. molitor* gut have been studied denoting the transient behavior of *Lb. plantarum* when the bacterial isolate was removed from the diet. The same trend, observed by (Storelli et al., 2018) in *Drosophila melanogaster*, highlighted the need to provide continuously the probiotic supplements in the diet for assuring the presence of the bacterial strain within the insect microbiota community. On the other hand, in **Chapter 5**, 16S sequencing analysis allowed to detect the presence of *P. pentosaceus* in all the larvae also fed with diets not containing the strain, conferring the specie a commensal status.

Moreover, not only the viable form but also dead cells and their products could be applied as health enhancers. The term postbiotics has been proposed for defining this category as "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021). In this context, components as bacteriocins or cell envelopes are taken into consideration and their singular impact on animal and insect immune responses have still to be unravelled. Bacteriocins application as preservatives in food products is used (Yi et al., 2022) and new perspectives could take into consideration insect feed bacteriocins supplementation (Pawlowska et al., 2012). In **Chapter 5**, the provision of the postbiotics (deactivated by heat tratement) preparation of *Lb. plantarum* WJB or *P. pentosaceus* KVL B19-01 has been tested in parallel with the vital strains. The insects were exposed to all the compounds present in the freeze-dried preparation (cell envelope and intra- and extracellular compounds) as purification of the components was not performed. The larvae fed on wheat bran containing *P. pentosaceus* vital and deactivated resulted in the same responses in terms of susceptibility to entomopathogens infections, increasing the interest in exploring the use of postbiotics for promoting insect health.

The spread of antimicrobial-resistant microorganisms resulted in the ban by the European Union to use antibiotics as growth enhancers and feed preservatives, which is expected to increase the application of probiotics as feed supplements (European Commission, 2018; Zommiti and Ferchichi, 2021).

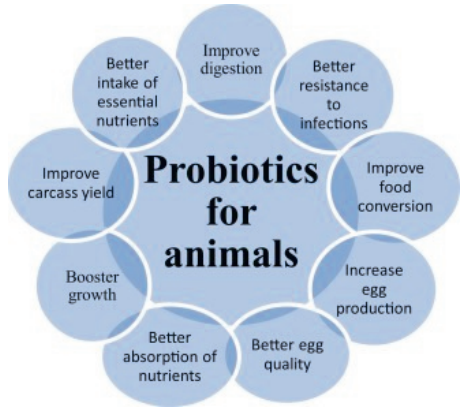

Figure 4 Main effects of probiotics on animal nutrition, health and breeding (Zommiti and Ferchichi, 2021).

Product formulation often implies processing methods as freeze-drying, lyophilization and microencapsulation along with prebiotic provision that assure cell viability for long periods, stockage at room temperature and rapid ingestion (Kiepś and Dembczyński, 2022).

The labelling of the products on the market should comprise the name of the microorganism strain(s), its load and the expiration date. Precise indications on stockage conditions and preparation procedures such as dilutions and quantities assure the effectiveness of the provision (U.S. Department of Health and Human Services Food and Drug Administration and Center for Food Safety and Applied Nutrition, 2018; Kiepś and Dembczyński, 2022). Despite all this information, several commercial products do not reflect the total viable count of bacteria reclaimed on the package, determining cleaner labelling and more stable production technologies such as nanoencapsulation (Weese, 2003; Kiepś and Dembczyński, 2022).

Gram-positive *Bacillus, Enterococcus, Lactobacillus, Lactococcus, Pediococcus*, and *Streptococcus* bacterial strains, as well as the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces*, are the most used probiotic agents in feed supplements in the European Union (Anadón et al., 2006; Gorrens et al., 2023; Savio et al., 2022).

Among the microorganisms with potential probiotic activities for insects, Bacilli, Gammaproteobacteria and Actinomyceta have been proposed for improving the quality and the efficiency of waste conversion of *Hermetia illucens* (Gorrens et al., 2023).

Probiotic provision in reared animals is often applied in the early life stages, for enhancing the immune system and the growth preventing the occurrence of pathologies and infections (Singh et al., 2023). In this thesis, the role of the timing and the length of probiotic provision has been taking into consideration in **Chapter 5** and **6**. In the first experiment, the provision was performed starting from the egg hatching until reaching a body mass of 20 mg, corresponding to the first larval instars. This period of the life cycle is the most vulnerable, characterized by higher mortality rates in case of entomopathogen infections (**Chapter 4**) and more sensitive to mechanical and environmental stressors.

In **Chapter 5**, *T. molitor* was exposed to *P. pentosaceus* and *Lb. plantarum* in the first larval instars until reaching a bodymass of 20 mg, which resulted in a protective role of *P. pentosaceus* against co-infections. The probiotic provision to early developmental stages might allow the insects to increase immune system responses. Similar protection against *B. thuringiensis* infections were previously reported with another bacterial species, *Enterococcus mundtii*, in the coleopteran *Tribolium castaneum*

(Grau et al., 2017). In detailed studies on the insect model *Galleria mellonella*, evidence was found for the ability of *Lactobacillus paracasei* to increase the protection against *Candida albicans* infections by increasing the insect's production of hemocytes and by upregulating genes encoding antifungal peptides (Ribeiro et al., 2017). However, the energy expenses of the activation of the immune pathway might lead to reduced performance or reduced fecundity (Grau et al., 2017).

The provision of *P. pentosaceus* to the yellow mealworm until reaching a body-mass suitable for commercial processing was conducted in **Chapter 6**. Acceleration of insect weight gain was observed after feeding on diets supplemented with the vital and inactivated *P. pentosaceus* in the first 40 days, for then presenting the same weight for all diets 10 days later. The results show different trends from the effect previously reported on *T. molitor* by Lecocq et al. (2021), where the insect weight gain was significantly improved by the probiotic source. Differences in diet formulation and *T. molitor* strain might have led to these different results.

The use of postbiotics in feed supplement formulation might result in increased insect performance and in easier handling and safer product storage (Kataria et al., 2009; Salminen et al., 2021; Weese, 2003). Even if conditions and dose of the probiotic is respected, it is possible that the insects do not respond as expected due to particular genetic or environmental factors (Villagra and Frías-Lasserre, 2020).

6. Immunological and metabolic pathways

Symbiont-host interactions trigger metabolic and immune pathways that modify the resistance to pathogen infection (Moret, 2006) and the energy available for storing nutrients, affecting growth performance (Grau et al., 2017).

Interactions between endosymbionts and insect host can lead to enhanced responses to pathogens, as it has been observed in flies colonized by *Spiroplasma* spp. (Son et al., 2021), or to symbiont microbial composition variation as observed in *Galleria mellonella* (Krams et al., 2017). Mechanisms behind these responses are the activation of the Toll immune pathway by interactions between bacterial cell surface components and gut immune receptors, resulting in higher levels of anti-microbial peptides (AMPs) production, as cecropins and defensins, $(Krams et al., 2017; Son et al., 2021; Vallet-Gely et al., 2008). In fact, insects reared in axenic conditions showed lower levels of immune gene expression, resulting in higher susceptibility to pathogens (Douglas, 2014). In **Chapter 5**, our results showed effects of beneficial symbiont provision as improved protection against pathogens whereas no effect on growth rates were found. These effects could be explained by the energy cost incurred by the insects maintaining higher levels of immune responses (Ardia et al., 2012; Dolezal et al., 2019). Immune challenged *Acheta domesticus* L. showed decreased fitness (Bascuñán-García et al., 2010) while in other mass-reared insects such as *Hermetia illucens* L., increased expression of immune genes did not affect fitness (Candian et al., 2023).

Providing different diets and beneficial symbionts at specific stages of the life cycle can modify the gene expression of the insect over an extended period, acting not only on performance but also on the immune system and the metabolic pathway, favouring the production of amino acids and lipids or antimicrobial peptides optimal for insect health and reproduction. The insect model *Drosophila melanogaster* has been subject to several studies related to microbial symbiont-host interactions (Consuegra et al., 2020; (Storelli et al., 2011); (Vallet-Gely et al., 2008). Several factors play a role in triggering the insect immune responses. For instance, pathogens such as *Xenorhabdus nematophila* and *Photorhabdus temperata* can interact and proliferate by reducing or inhibiting the activity of the phospholipase A2 (PLA 2), produced in the insect fat body and responsible for hydrolyzing fatty acids

from the *sn-2* position of phospholipids leading to the production of eicosanoids. These molecules are essential for insect immune responses to pathogen infections (Stanley-Samuelson et al., 1991) assuring haemocyte encapsulation and prophenoloxidase activation (Kim et al., 2005). Beneficial microbes such as *Lb. plantarum* WJB allowed gnotobiotic insects to develop in sub-optimal nutritional conditions, acting on the Tor signal and favouring larval development and shortening the duration of the life stage (Consuegra et al., 2020; Storelli et al., 2011).

Insect microbial exposure for a short period of time could also result in increased protection against pathogens due to immune system responses activated by the interactions between the microorganism and the host receptors (Muhammad et al., 2019). Immune priming strategies could be considered in breeding programs to decrease the incidence of pathogen infection in mass-reared insect populations. Strategies could imply providing deactivated probiotics such as *P. pentosaceus* (**Chapter 5**) or pathogens such as *Bacillus thuringiensis* (Moret, 2006) for activating genes related to immune responses. However, these activations could also result in lower insect performance.

Exploring the yellow mealworm transcriptome after feed supplementation with beneficial symbiont (pre-or probiotic) would for instance provide information on expression of genes related to immune response pathways, and then also yield information on insect metabolic energy expenses in such conditions. Moreover, metabolomic approaches could supply more information related to host-symbiont relationships (Nagana Gowda and Raftery, 2023). The number of individuals required for such studies is high in order to obtains robust results but considering the high reproduction rate and short life cycles of most insect species, makes it feasible to increase the number of replicates in a given experiment.

Conclusions and Perspectives

Insect mass-rearing facilities are characterized by environmental conditions that could lead to the occurrence of entomopathogen infections. Findings reported in this thesis suggest that both probiotics and diet play a role in maintaining *T. molitor* health. Indeed, probiotic provision to early insect life stages might prevent and reduce the incidence of infections. Moreover, indications were found that probiotic provision over a longer period of larval development improved larval growth on low-protein diets, suggesting an added value of probiotic supplementation. Feed formulation with prebiotic and probiotic acting as synbiotic could allow the preservation of insect health in a circular economy context, where the use of organic side streams as feed resources might increase the risk of pathogens infection. The possibility to provide postbiotics as feed supplements allow the use of the inactivated form of previously tested bacterial species and/or their metabolites. Meanwhile, considering the cost of feed, adding a high value protein source such as dried egg white could be more convenient or cost-effective than providing bacterial sources. An integrated approach that takes into consideration omics-based technologies such as transcriptomics, proteomics and metagenomics could help in identifying the optimal conditions for assuring insect health and a sustainable and high-quality production.

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Summary

Most mass reared insect can be exposed to entomopathogens with risk of infections, while the impact will depend notably on the environmental conditions, the nutritional feed quality and the microbiota composition of the insects. The yellow mealworm, *Tenebrio molitor*, is an edible insect mainly reared on cereal-based diets and other agricultural by-products, which can potentially expose the insect to entomopathogens applied as crop protection agents, like the bio-pesticides as the bacterium *Bacillus thuringiensis* or fungi as *Metarhizium brunneum*. In this thesis question related to the evaluation on the impact of insect feed quality and the addition of two bacterial probiotics (alive/killed) on larval growth and susceptibility to two entomopathogens were addressed by performing *in vivo* experiments with a *B. thuringiensis* strain active on Coleopteran larva. The experimental protocols were based on free feeding method and by using high larval densities in order to be close to the mass rearing environment. A second aspect considered analysis on the bacterial microbiota composition and abundance in order to measure the impact on the microbiota and the persistence of the two probiotics and the bacterial pathogen. A third aspect aimed to investigate to what level the feed composition (low and high protein content) and probiotic addition can influence larval growth and the protein and lipid composition of the larva.

Chapter 1 provides a general introduction on insect consumption, focusing on *T. molitor* life cycle and its applications in a circular economy context. The insect mass rearing for feed and food purposes along with a brief description of entomopathogens infections highlighting the importance of following Good Manufactural Practices and HACCP guidelines for reducing their incidence. *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* and *Metarhizium brunneum* biology and insect infection rutes are then described for introducing the reader to the experimental chapters 4 and 5. Beneficials microbes and their applications for preserving insect health are then introduced along with two sections dedicated to *P. pentosaceus* and *Lb. plantarum*. Their biology and technological applications are described along with reasons related to their selection for this thesis. The chapter contains objectives and outlines of the thesis, offering an overview on the content of the next sections.

In **Chapter 2**, which is a published review paper, we reviewed insect-microbiota interactions, focusing on the role of probiotics in maintaining insect health, nutrient provision and in shaping host behavior, pointing out the complexity of the system. Examples on several insect species demonstrate that when specific microorganisms are provided as a dietary supplement, insect reproduction, food conversion and growth are enhanced and health is improved in cases of nutritional deficiency or pathogen infection. The section contains definitions of probiotics and prebiotics and their general use in insects reared for food and feed is addressed, listing the bacterial and fungal already tested in the insect species of main interest in the mass rearing sector as *Tenebrio molitor*, *Hermetia illucens* and *Bombyx mori* and the products already present on the market. Moreover, the chapter contains methods for selection of probiotic bacterial strains and discussion on methods as isolation and *in vivo* and *in vitro* test for proving the probiotic properties, following the current legislation. Finally, at the chapter, perspectives on probiotic applications in mass-rearing facilities are addressed.

In **Chapter 3**, also a published review paper, the focus is set on *Tenebrio molitor* and its beneficial and harmful symbionts in mass rearing systems. High densities and environmental conditions result in conditions of pathogens spoilage, increasing the importance to apply efficient detection methods and

preventive actions for reducing the risk of pathogens occurrence in the rearing systems. On the other hand, the presence of beneficial symbionts can lead to improved performances and protection against pathogens. A list of yellow mealworm beneficial symbionts is provided along with the effects on its performances and nutritional content. Knowing the importance of symbionts in preserving insect health, microbial control strategies and beneficial symbionts applications are addressed at the end of the chapter.

In **Chapter 4**, the impact of *Bacillus thuringiensis* sv *morrisoni* biovar *tenebrionis* on several instars of yellow mealworm larvae is investigated. The inclusion of by- and side-products of cereal and vegetable production in the feed of yellow mealworm increases the probability of infection by *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* (Btt), an entomopathogen used in the biological control of coleopteran pest species. The experimental set up was designed mimicking the mass rearing conditions reproducing larval density and by infecting the larvae with the Btt spore-toxin suspension by free feeding on contaminated wheat bran for 72 hours. The study contains results on Btt impact on yellow mealworm survival and growth and its persistence in larvae and frass. The results highlight the direct correlation between larval body mass and entomopathogen susceptibility, larvae of higher mass being less susceptible. Effects on growth and feed conversion ratio of survivors showed significant impact for low mass larvae while less or no effect was recorded for higher mass larva. Btt was still recovered from larvae and frass 14 days after feeding with non-contaminated wheat bran, indicating a certain level of persistence, and therefore a potential risk in rearing facilities.

In **Chapter 5**, *in vivo* bacterial probiotics supplementation in two feed formulations differing in protein content were investigated for observing their effects on *T. molitor* early stages larvae growth, microbial composition and susceptibility to entomopathogen infection. Two probiotic bacterial species, *Pediococcus pentosaceus* KVL B19-01 and *Lactobacillus plantarum* WJB were added in vital and deactivated form to wheat bran with and without egg-white powder, from egg hatching to early larval developmental stages. Twenty mg larvae were then exposed over 72 h to *B. thuringiensis, M.brunneum* or their combination applying the free feeding infection method used in the previous chapter. Larval survival and growth were recorded for 14 days. The results showed that the provision of *P. pentosaceus* in the wheat bran led to increased survival in case of co-infection, suggesting a protective role in its vital and deactivated form. On the other hand, larvae fed with *Lactobacillus plantarum* presented increased weight gain but not increased survival, suggesting a different allocation of energies in the metabolic pathways. In the diet formulated with dried egg white, no effect on larval survival was found in the presence of the two pathogens, suggesting a protective role of diet components against infections. Overall, the presence of the entomopathogens was the significant variable that affected the larval growth. Along the analysis on insect performances, the bacterial microbiota composition by 16S rDNA sequencing pre-pathogen exposure (day 0), and at days 3 and 14 after inoculation with the pathogens was analysed. The objectives were to study the pathogens and probiotic persistence and the effects of diet formulation on microbial dynamics. In all the samples, *Pediococcus pentosaceus* was detected from day 0 through-out the assay at the species level and *Lb. plantarum* was not detectable. The diet containing dried egg white showed no differences in relation to microbial richness after probiotic provision, suggesting a potential antimicrobial action of lysozymes on the insect microbial community. In the wheat bran diet, the effect of vital or deactivated probiotic provision was observed in terms of genera abundances and richness in the two diets. At day14, the bacterial community composition of the larvae was similar in all treatments, indicating that the pathogen did not establish at a detectable level in the insect.

In **Chapter 6,** we investigated on the effects of probiotic provision and feed composition on *Tenebrio molitor* larvae performance, protein content and lipid composition. The selection of the probiotic

isolate *Pediococcus pentosaceus* KVL B19-01 was based on results of the previous experimental chapter 5. In this study, the effects of probiotic provision and feeds composition were recorded when the larval weight had reached 120 mg. The experimental diets based on wheat bran, as in the former assays, were formulated by reducing the protein content by including potato starch. The growth was similar on all diets. Semi-quantitative analysis of the fatty acids profile of *T. molitor* larvae were performed by GC-FID. The results highlight how deactivated probiotics provision improved fatty acids productions and storage in the insect body of larvae reared in low nutrient diet. Overall, the chemical larval composition was influenced by the diet composition and not by probiotic provision. However, the provision of the probiotic strain resulted in larvae with FAMEs profiles characterized by an increment in MUFAs and PUFAs. The results highlight the possibility to obtain larvae with a fatty acid profile that is nutritionally more favorable for humans and animals. The discussion contains suggestions on a commercial potential interest in supplementing the probiotic strain to obtain insects with a specific nutritional profile.

In **Chapter 7**, I discuss the overall results of this thesis, in the context of insect a mass rearing and new technologies developed during these years. In a first introduction section I discuss the sustainability of the yellow mealworm production for food and feed purposes, taking into consideration environmental, economic, social aspects. In the next section, I discuss the structure of the mass rearing environment and the variables that affect insect health. Providing an overview on the actual status of safety requirements and insights and perspective on approaches that might be applied for implementing already existing good manufactural practices for assuring insect and consumer safety. Another section is dedicated to insect nutrition and its role in shaping insect nutrient composition, denoting the importance of feed protein content in maintaining insect metabolic pathways and lipids production. The last part of the discussion is dedicated to probiotic application as feed supplements. Their effects on animal immune responses and metabolism are briefly indicated and discussed along with the results obtained from my experimental work, in the previous chapters. Perspectives based on omics-analyses approaches are suggested for unravelling host-microbiota and diet interactions.

Resumé

La plupart des insectes élevés en masse peuvent être exposés à des entomopathogènes avec des risques d'infection, et leur impact dépendra notamment des conditions environnementales, de la qualité nutritionnelle de l'alimentation et de la composition du microbiote des insectes. Le ver de farine jaune, *Tenebrio molitor*, est un insecte comestible élevé principalement avec des régimes à base de céréales et d'autres sous-produits agricoles, ce qui peut potentiellement exposer l'insecte à des entomopathogènes appliqués comme agents phytosanitaires, par exemple les biopesticides comme la bactérie *Bacillus thuringiensis* ou des champignons comme *Metarhizium brunneum*. Dans cette thèse, la question liée à l'évaluation de l'impact de la qualité de l'alimentation des insectes et de l'ajout de deux probiotiques bactériens (vivants/tués) sur la croissance larvaire et la sensibilité à deux entomopathogènes a été abordée en réalisant des expériences *in vivo* avec une souche de *B. thuringiensis* active sur les larves de coléoptères. Les protocoles expérimentaux étaient basés sur une méthode d'alimentation libre et sur l'utilisation de densités larvaires élevées afin d'être proches de l'environnement d'élevage de masse. Un deuxième aspect a porté sur l'analyse de la composition et de l'abondance du microbiote bactérien afin de mesurer l'impact sur le microbiote et la persistance des deux probiotiques et du pathogène bactérien. Un troisième aspect visait à étudier dans quelle mesure la composition alimentaire (teneur faible et élevée en protéines) et l'ajout de probiotiques peuvent influencer la croissance larvaire et la composition protéique et lipidique des larves.

Le **Chapitre 1** fournit une introduction générale sur la consommation d'insectes, en se concentrant sur le cycle de vie de *T. molitor* et ses applications dans un contexte d'économie circulaire. L'élevage en masse d'insectes à des fins alimentaires et fourragères ainsi qu'une brève description des infections entomopathogènes soulignant l'importance de suivre les bonnes pratiques de fabrication et les directives HACCP pour réduire leur incidence. *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* et *Metarhizium brunneum*, la biologie et les méthodes d'infection par les insectes sont ensuite décrites pour présenter au lecteur les chapitres expérimentaux 4 et 5. Les microbes bénéfiques et leurs applications pour préserver la santé des insectes sont ensuite introduits ainsi que deux sections dédiées à *P. pentosaceus* et *Lb. plantarum*. Leur biologie et applications technologiques sont décrites ainsi que les raisons liées à leur utilisation pour cette thèse. Le chapitre contient les objectifs et les grandes lignes de la thèse, offrant un aperçu du contenu des sections suivantes.

Dans le **Chapitre 2**, nous avons examiné les interactions insectes-microbiote, en nous concentrant sur le rôle des probiotiques dans le maintien de la santé des insectes, de leur apport en nutriments et dans la formation du comportement de l'hôte, soulignant la complexité du système. Des exemples sur plusieurs espèces d'insectes démontrent que lorsque des micro-organismes spécifiques sont fournis sous forme de complément alimentaire, la reproduction, la conversion alimentaire et la croissance des insectes sont améliorées et la santé est améliorée en cas de carence nutritionnelle ou d'infection pathogène. La section contient les définitions des probiotiques et des prébiotiques, leur utilisation générale chez les insectes élevés pour l'alimentation humaine et animale est abordée, répertoriant les bactéries et les champignons déjà testés sur les espèces d'insectes d'intérêt principal dans le secteur de l'élevage de masse comme *Tenebrio molitor*, *Hermetia illucens* et *Bombyx mori* et les produits déjà présents sur le marché. De plus, le chapitre contient des méthodes de sélection de souches bactériennes probiotiques et une discussion sur des méthodes telles que l'isolement et les tests *in vivo* et *in vitro* pour prouver les propriétés probiotiques, conformément à la législation en vigueur. Enfin, dans ce chapitre, les perspectives sur les applications des probiotiques dans les installations d'élevage de masse sont abordées.

Dans le **Chapitre 3**, également un article de synthèse publié, met l'accent sur *Tenebrio molitor* et ses symbiotes bénéfiques et nuisibles dans les systèmes d'élevage de masse. Leurdensité élevée et les conditions environnementales entraînent des conditions de détérioration par les agents pathogènes, ce qui accroît l'importance d'appliquer des méthodes de détection efficaces et des actions préventives pour réduire le risque d'apparition d'agents pathogènes dans les systèmes d'élevage. D'un autre côté, la présence de symbiotes bénéfiques peut conduire à des performances améliorées et à une protection contre les agents pathogènes. Une liste des symbiotes bénéfiques du ver de farine jaune est fournie ainsi que leurs effets sur ses performances et leurcontenu nutritionnel. Connaissant l'importance des symbiotes dans la préservation de la santé des insectes, les stratégies de contrôle microbien et les applications bénéfiques des symbiotes sont abordées à la fin du chapitre.

Dans le **Chapitre 4**, l'impact de *Bacillus thuringiensis* sv. *morrisoni* biovar *tenebrionis* sur plusieurs stades larvaires de vers de farine jaune est étudié. L'inclusion de sous-produits et de sous-produits de la production céréalière et maraîchère dans l'alimentation du ver de farine jaune augmente la probabilité d'infection par *Bacillus thuringiensis* sv. morrisoni biovar *tenebrionis* (Btt), un entomopathogène utilisé dans la lutte biologique contre les espèces nuisibles de coléoptères. Le dispositif expérimental a été conçu en imitant les conditions d'élevage en masse, reproduisant la densité larvaire et en infectant les larves avec la suspension spores-toxines Btt en se nourrissant librement de son de blé contaminé pendant 72 heures. L'étude contient des résultats sur l'impact du Btt sur la survie et la croissance du ver de farine jaune ainsi que sur sa persistance dans les larves et les excréments. Les résultats mettent en évidence la corrélation directe entre la masse corporelle des larves et la susceptibilité aux entomopathogènes, les larves de masse plus élevée étant moins sensibles. Les effets sur la croissance et le taux de conversion alimentaire des survivants ont montré un impact significatif pour les larves de faible masse, tandis que moins ou pas d'effet a été enregistré pour les larves de masse plus élevée. Le Btt était encore récupéré des larves et des excréments 14 jours après avoir été nourri avec du son de blé non contaminé, ce qui indique un certain niveau de persistance, et donc un risque potentiel dans les installations d'élevage.

Dans le **Chapitre 5**, la supplémentation en probiotiques bactériens *in vivo* dans deux formulations alimentaires différant par leur teneur en protéines a été étudiée afin d'observer leurs effets sur la croissance des larves de *T. molitor* aux premiers stades, la composition microbienne et la sensibilité à l'infection entomopathogène. Deux espèces bactériennes probiotiques, *Pediococcus pentosaceus* KVL B19-01 et *Lactobacillus plantarum* WJB, ont été ajoutées sous forme vitale et désactivée au son de blé avec et sans poudre de blanc d'œuf, depuis l'éclosion des œufs jusqu'aux premiers stades de développement larvaire. Des larves de vingt mg ont ensuite été exposées pendant 72 h à *B. thuringiensis, M. brunneum* ou à leur combinaison en appliquant la méthode d'infection par alimentation libre utilisée dans le chapitre précédent. La survie et la croissance des larves ont été enregistrées pendant 14 jours. Les résultats ont montré que l'apport de *P. pentosaceus* dans le son de blé entraînait une augmentation de la survie en cas de co-infection, suggérant un rôle protecteur sous sa forme vitale et désactivée. D'autre part, les larves nourries avec *Lb. plantarum* ont présenté une prise de poids accrue mais pas une survie accrue, suggérant une répartition différente des énergies dans les voies métaboliques. Dans le régime alimentaire formulé avec du blanc d'œuf séché, aucun effet sur la survie des larves n'a été observé en présence des deux agents pathogènes, ce qui suggère un rôle protecteur des composants du régime alimentaire contre les infections. Dans l'ensemble, la présence d'agents entomopathogènes était la variable significative affectant la croissance larvaire. Parallèlement à l'analyse des performances des insectes, la composition du microbiote bactérien a été analysée par séquençage de l'ADNr 16S avant l'exposition aux agents pathogènes (jour 0), ainsi qu'aux jours 3 et 14 après l'inoculation des agents pathogènes. Les objectifs étaient d'étudier les pathogènes et la persistance des probiotiques ainsi que les effets de la formulation du régime alimentaire sur la dynamique

microbienne. Dans tous les échantillons, *P. pentosaceus* a été détecté dès le jour 0, tout au long du test au niveau de l'espèce tandis que *Lb. plantarum* n'était pas détectable. Le régime alimentaire contenant du blanc d'œuf séché n'a montré aucune différence en ce qui concerne la richesse microbienne après l'apport de probiotiques, ce qui suggère une action antimicrobienne potentielle des lysozymes sur la communauté microbienne des insectes. Dans le régime alimentaire au son de blé, l'effet de l'apport de probiotiques vitaux ou désactivés a été observé en termes d'abondance et de richesse des genres dans les deux régimes. Au jour 14, la composition de la communauté bactérienne des larves était similaire dans tous les traitements, ce qui indique que l'agent pathogène ne s'est pas établi à un niveau détectable chez l'insecte.

Avec le **Chapitre 6**, nous avons étudié les effets de l'apport de probiotiques et de la composition des aliments sur les performances des larves de *T. molitor*, leur teneur en protéines et leur composition en lipides. La sélection de l'isolat probiotique *Pediococcus pentosaceus* KVL B19-01 était basée sur les résultats du chapitre expérimental précédent 5. Dans cette étude, les effets de l'apport en probiotiques et de la composition des aliments ont été enregistrés lorsque le poids des larves avait atteint 120 mg. Les régimes expérimentaux à base de son de blé, comme dans les essais précédents, ont été formulés en réduisant la teneur en protéines en incluant de la fécule de pomme de terre. La croissance était similaire pour tous les régimes. Une analyse semi-quantitative du profil des acides gras des larves de *T. molitor* a été réalisée par GC-FID. Les résultats mettent en évidence comment l'apport de probiotiques désactivés a amélioré la production et le stockage d'acides gras dans le corps des insectes des larves élevées avec un régime pauvre en nutriments. Dans l'ensemble, la composition chimique des larves était influencée par la composition du régime alimentaire et non par l'apport de probiotiques. Cependant, l'apport de la souche probiotique aux larves fait apparaitre des profils en FAME caractérisés par une augmentation des acides gras mono et poli insaturés (AGMI et AGPI). Les résultats mettent en évidence la possibilité d'obtenir des larves avec un profil en acides gras nutritionnellement plus favorable pour l'homme et l'animal. La discussion contient des suggestions sur un intérêt commercial potentiel dans la supplémentation en souche probiotique pour obtenir des insectes ayant un profil nutritionnel spécifique.

Dans le **Chapitre 7**, je discute les résultats globaux de cette thèse, dans le contexte de l'élevage de masse d'insectes et des nouvelles technologies développées au cours de ces années. Dans une première section d'introduction, j'évoque la durabilité de la production de vers de farine jaune à des fins alimentaires et fourragères, en tenant compte des aspects environnementaux, économiques et sociaux. Dans la section suivante, je discute de la structure de l'environnement d'élevage de masse et des variables qui affectent la santé des insectes. Fournir un aperçu de l'état actuel des exigences de sécurité, ainsi que des informations et une perspective sur les approches qui pourraient être appliquées pour mettre en œuvre les bonnes pratiques de fabrication déjà existantes pour garantir la sécurité des insectes et des consommateurs. Une autre section est consacrée à la nutrition des insectes et à son rôle dans la composition des nutriments des insectes, soulignant l'importance de la teneur en protéines alimentaires dans le maintien des voies métaboliques des insectes et de la production de lipides. La dernière partie de la discussion est consacrée à l'application des probiotiques comme compléments alimentaires. Leurs effets sur les réponses immunitaires et le métabolisme des animaux sont brièvement indiqués et discutés ainsi que les résultats obtenus lors de mes travaux expérimentaux. Des perspectives basées sur des approches d'analyses omiques sont suggérées pour démêler les interactions hôte-microbiote et régime alimentaire.

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La Danse - Henri Matisse (1910)

About the author

Carlotta was born on the 2nd of March 1994 in Turin, Italy, where she grew up with her parents and family. After completing a High School scientific diploma, she continued her studies at the University of Turin ad the department of Agricultural, Forest and Food Sciences (DISAFA) where she studied Food Science and obtained her bachelor's degree in 2016, where she wrote a manuscript focused on the integration of insect consumption in occidentals' diets. Her fascination for insects and their importance for the environment grew during an internship related to beekeeping, where bees' behavior and their interactions, with several trophic ecosystem levels, captured her attention.

She then decided to continue her studies in Food Science and Technologies at the University of Turin, still at DISAFA, where she obtained her Master's degree in 2019. During the two years, she had the chance to participate to one European Union Erasmus + internship at the Danish Technological Institute, developing a food product based on insect proteins, and a second European Union Erasmus + studying period of 6 month at the Ghent University. During her Master, courses related to food sustainability and insect production further increased her interest in insect's application in a circular economy. During her internship at the Department of General and Applied Entomology of the University of Turin (DISAFA), Carlotta had the opportunity to work under the supervision of Valentina Candian and Rosemarie Tedeschi on the impact of diet on immune gene expression in the black soldier fly larvae, an insect species with great potential for resources reallocation.

After obtaining her Master's degree, she worked as early researcher for two months before starting her PhD within the European funded joint Doctoral program "Insect Doctors". This vacancy represented the perfect opportunity for developing a project focused on insect health and microbial interactions of the yellow mealworm, an insect species nowadays produced for food and feed purposes. In 2020 Carlotta started her PhD under the supervision of Christina Nielsen-LeRoux at INRAE, Jouy en Josas, France, of Joop van Loon at the Laboratory of Entomology, Wageningen, the Netherlands and the guidance of Annette Bruun Jensen at the University of Copenhagen. During her PhD, Carlotta studied the effects of feed and bacterial probiotics on yellow mealworm performances, microbial community composition and resistance to pathogens. All major results are presented in the thesis. Conference participation, supervising of two students and sharing ideas and opinions with colleagues, were insightful and energizing parts of the PhD training period.

The PhD is getting to the end and her ambition is to follow her the research career by contributing to the unraveling of microorganisms-host dynamics.

List of publications

Anna Ruth Slowik, Pascal Herren, Edouard Bessette, Fang Shiang Lim, Luis Hernández-Pelegrín, Carlotta Savio (2023). **Harmful and beneficial symbionts of *Tenebrio molitor* and their implications for disease management.** Journal of Insects as Food and Feed, 1-16. https://doi.org/10.3920/JIFF2022.0171

Carlotta Savio, Loretta Mugo-Kamiri and Jennifer K. Upfold (2022) **Bugs in Bugs: The Role of Probiotics and Prebiotics in Maintenance of Health in Mass Reared Insects.** Insects 13(4), 376. https://doi.org/10.3390/insects13040376

Valentina Candian, Carlotta Savio, Marco Meneguz, Laura Gasco, Rosemarie Tedeschi (2023), **Effect of the rearing diet on gene expression of antimicrobial peptides in *Hermetia illucens* (Diptera: Stratiomyidae**). Insect Science. https://doi.org/10.1111/1744-7917.13165

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review/project proposal (10.5 ECTS)

- Bugs in bugs: the role of probiotics and prebiotics in health maintenance in mass reared insects
- Harmful and beneficial symbionts of *Tenebrio molitor* and their implications for disease management

Post-graduate courses (13.2 ECTS)

- Concepts and terms in insect pathology and their placement in the general context of diseases and health; UCPH (2020)
- Bioinformatique par la pratique; INRAE (2020)
- Laboratory methods (diagnostics, bio-assays) in insect pathology across organisms; INRAE and CNRS (2021)
- The value and pitfalls of metagenomics in pathogen detection and discovery; INRAE (2021)
- Mixtures and Combined Stressors; use of multi-stressor theory for invertebrate function based end points; CESAM (2021)
- Insects as feed; PE&RC (2022)
- Transdisciplinary training on topics concerning Insect as food and feed; INRAE and CNRS (2022)

Deficiency, refresh, brush-up courses (2.4 ECTS)

- Basic statistics; PE&RC (2020)
- Introduction to R statistical software; INRAE (2020)

Laboratory training and working visits (4.1 ECTS)

- Methods in insect pathology with focus on entomopathogenic fungi; UCPH (2020)
- BSTS better safe than sorry; WUR (2021)

Invited review of journal manuscripts (5 ECTS)

- Foods: effect of probiotics preparations on gut microbiota metabolism on an in vitro fermentation model (2022)
- Bacteria: isolation and identification of lactic acid bacteria from indigenous African vegetables (2022)
- Applies Sciences: optimisation of thermal process on prebiotic properties of a seaweed (2022)
- Journal Insect as Food and Feed: connection between immune responses and performances of *Opisthoplatia orientalis* at low temperatures (2022)

- Journal Insect as Food and Feed: connection between immune responses and performances of *Opisthoplatia orientalis* at low temperatures: use of probiotics for implementing the insect breeding (2022)

Competence, skills and career-oriented activities (13.4 ECTS)

- Scientific publishing; Wageningen University (2020)
- Journal Club ABIES communication et médiation scientifique; EBIES (2020)
- Workshop on the Individual Research Projects and the Collaborations within and across WPs; Wageningen University (2020)
- Reviewing a scientific manuscript; Wageningen University (2020)
- Développer des relations professionnelles constructives; Univ Paris Saclay (2021)
- How to write highly cited papers; UK Centre for Ecology and Hydrology (2021)
- Improving Grant Writing Skills in English (online course); Univ Paris Saclay (2022)
- Approches, méthodes et outils inédits de renforcement de l'employabilité des docteurs; Univ Paris Saclay (2022)
- Parcours Entrepreneuriat Deeptech – Module « Docteur en Start-up »; Univ Paris Saclay (2022)
- Propriété intellectuelle: droit des marques, AOP, droit des brevets; Univ Paris Saclay (2022)

Scientific integrity/ethics in science activities (1.3 ECTS)

- Research integrity in scientific professions; ABIES /Univ Paris Saclay (2020)
- Science integrity; WUR (2022)

PE&RC Annual meetings, seminars and the PE&RC retreat (0.5 ECTS)

- PE&RC Day (2022)
- Symposium: the insider's guide to science communication (2022)
- Minlab theatre performance (2022)

Discussion groups/local seminars or scientific meetings (6.4 ECTS)

- CNiE Conference (2020)
- Workshop on individual research projects (2020)
- JDD ABIES (2020)
- Journée d'accueil des nouveaux doctorants (2020)
- Seminars at Micalis (2020-2023)
- Entomology day (2022)
- AGSx Virtual symposium (2022)
- Insect doctors and stakeholders symposium (2022)

International symposia, workshops and conferences (4.7 ECTS)

- AFFIA Conference; oral presentation; online (2020)
- Society for invertebrate pathology annual conference; poster presentation; online (2021)
- European PhD network insect science XII annual meeting; oral presentation; online (2021)
- Insect as food and feed; oral presentation; London, UK (2022)
- Society for invertebrate pathology annual conference; oral presentation; online (2022)
- 73rd Annual conference of animal science; oral presentation; Porto, Portugal (2022)
- Entomological society of America annual meeting; online (2022)
- 9th Conference of beneficial microbes; Amsterdam, the Netherlands (2022)
- Asian insect industry & research forum; online (2023)

- Society for invertebrate pathology annual conference; oral presentation; Washington, USA (2023)

Societally relevant exposure (1 ECTS)

- Salon international de l'agriculture; Paris, France (2023)

Lecturing/supervision of practicals/tutorials (3 ECTS)

- Laboratory methods (2021)
- Insect as food and feed (2022)

BSc/MSc thesis supervision (2 ECTS)

- Effects of probiotics and feed on *Tenebrio molitor* microbial composition

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