UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN

SCIENZE DELLA VITA E BIOTECNOLOGIE

XXXIII CICLO

Insect immune system: a tool for biotechnological applications

Il sistema immunitario degli insetti: uno strumento per applicazioni biotecnologiche

Docente guida: Prof. **MAGDA DE EGUILEOR**
Tutor: Prof. **GIANLUCA TETTAMANTI**

Tesi di dottorato di:
AURORA MONTALI
Matr. 709053

Dip. Biotecnologie e Scienze della Vita - Università degli Studi dell'Insubria

Anno accademico 2019-2020

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SUMMARY

Insects represent nearly 90 % of the animal beings on Earth and this *taxon* contains more species than any other groups of organisms. They can live in different ecological niches and are in continuous contact with several types of microorganisms. For this reason, they have evolved advanced immune response mechanisms, whose knowledge can become the source of biotechnological applications. Nowadays, "insect biotechnology", also known as "yellow biotechnology", is very popular and aims at developing and manufacturing different products in medicine, feed industry, and pest management based on insects. In this context, the silkworm, *Bombyx mori*, and the black soldier fly (BSF), *Hermetia illucens,* that belong to the orders of Lepidoptera and Diptera, respectively, play an important role. More in detail, *B. mori* has been used for centuries in sericulture, and represents a model organism among Lepidoptera for life science, pharmaceutical, and biotechnological studies. On the other hand, *H. illucens* is a natural decomposer, whose larvae have a high nutritional value, which makes them particularly suitable for the production of feedstuff.

These two insects were used as model organisms for this PhD project. In particular, in this thesis two different biotechnological applications based on the immune system of insects are presented.

The first chapter deals with the use of cellular and humoral immune markers to develop an alternative infection model for the screening of new antimicrobial compounds. Interestingly, *B. mori* can also be reared at 37 °C, thus mimicking human physiological conditions. We demonstrate that *B. mori* larvae represent an excellent candidate for the initial screening of glycopeptide antibiotics. Therefore, the use of this insect can be taken into consideration and included in

preclinical tests to reduce the time required for the analyses and also the number of mammals used for this purpose.

In the second chapter the characterization of the immune response of BSF larvae is reported. As mentioned above, this insect can be used to reduce organic waste and, at the same time, to obtain proteins and lipids for the production of feedstuff. Here, the cellular and the humoral immune responses were investigated in larvae infected with Gram-positive and Gram-negative bacteria. This study provides a starting point for the future manipulation of BSF immune response. In particular, by acting on environmental factors as the composition of the rearing substrate or the rearing temperature, the health status of the larvae and their pathogen resistance during mass rearing procedures could be improved and, consequently, the value of feedstuff products derived from this insect increased.

The results here presented can be used as a platform for future biotechnological studies aimed at developing two different final products: the former exploitable in the field of drug research, while the second will contribute to improve circular economy.

RIASSUNTO

Gli insetti rappresentano quasi il 90 % degli esseri animali sulla Terra ed inoltre a questo *taxon* appartengono più specie di qualsiasi altro gruppo di organismi. Sono in grado di occupare diverse nicchie ecologiche e si trovano in continuo contatto con diversi tipi di microrganismi. Per questo motivo, hanno sviluppato una sofisticata risposta immunitaria, la cui conoscenza può diventare fonte di applicazioni biotecnologiche. Al giorno d'oggi, le biotecnologie entomologiche, note anche come "*yellow biotechnology*", sono molto popolari e mirano a sviluppare diversi prodotti utili in medicina, per le industrie mangimistiche ed il controllo di insetti dannosi. In questo contesto, il baco da seta *Bombyx mori* e la mosca soldato nera (BSF), *Hermetia illucens*, appartenenti rispettivamente agli ordini dei Lepidotteri e Ditteri, giocano un ruolo importante. Nel dettaglio, *B. mori* è stato utilizzato per secoli nel campo della sericoltura ed è diventato un organismo modello tra i lepidotteri per studi in ambito biologico, farmaceutico e biotecnologico. *H. illucens* è invece considerato un decompositore naturale, le cui larve hanno un elevato valore nutritivo che le rende particolarmente adatte alla produzione di mangimi.

Questi due insetti sono stati usati come organismi modello per il presente progetto di dottorato. In particolare, in questa tesi vengono presentate due diverse applicazioni biotecnologiche basate sul sistema immunitario degli insetti.

Il primo capitolo tratta dell'utilizzo di marcatori immunitari cellulari e umorali per lo sviluppo di un modello di infezione alternativo per lo screening di nuovi composti antimicrobici. È interessante notare che *B. mori* può essere allevato a 37 °C, permettendo così di operare in condizioni simili a quelle fisiologiche umane. In questo lavoro, abbiamo dimostrato che le larve di *B. mori* rappresentano un

ottimo candidato per lo screening iniziale di antibiotici glicopeptidici. Pertanto, l'utilizzo di questo insetto può essere preso in considerazione e incluso nei test preclinici per ridurre sia le tempistiche necessarie per le analisi che il numero di mammiferi utilizzati a tale scopo.

Nel secondo capitolo viene riportata la caratterizzazione della risposta immunitaria delle larve di BSF. Come accennato in precedenza, questo insetto può essere utilizzato per ridurre i rifiuti organici e, allo stesso tempo, ottenere proteine e lipidi impiegabili per la produzione di mangimi. Le risposte cellulari e umorali sono state studiate in larve infettate con batteri Gram-positivi e Gram-negativi. Questo studio fornisce una piattaforma di conoscenza per effettuare future manipolazioni della risposta immunitaria di *H. illucens*. In particolare, agendo su fattori ambientali come il substrato e la temperatura di allevamento, si potrebbero migliorare la salute delle larve e la loro resistenza agli agenti patogeni durante le procedure di allevamento massale, aumentando di conseguenza il valore dei mangimi derivati da questo insetto.

I risultati qui presentati possono essere utilizzati come punto di partenza per futuri studi biotecnologici finalizzati allo sviluppo di due diversi prodotti finali: il primo sfruttabile nel campo della ricerca sui farmaci, mentre il secondo permetterà lo sviluppo di processi di interesse per l'economia circolare.

INTRODUCTION

Multicellular organisms come into contact with several kind of pathogens during their life (e.g., bacteria, virus, fungi, protozoa, and parasites). The defense mechanisms used by the host against pathogens are based on behavioral processes, as well as physical, biochemical, and chemical barriers (Siva-Jothy et al., 2005).

In insects, infections can occur via the integument, by ingestion, or respiration. In this context, the cuticle and the epithelial cells represent the first line of defense against pathogens and parasites (Gillespie et al., 1997). In particular, the cuticle, which is composed of an epicuticle and a procuticle, is a thick, strong, sclerotized, and flexible barrier able to prevent or slow down the entry of pathogens (St. Leger, 1991; Kavanagh and Reeves, 2004). Invaders can also enter the insect through the spiracles and tracheal system (Hajek and Leger, 1994; Aikawa and Togashi, 2000), or through the gut following ingestion. Also in this organ the defense of the insect relies on the presence of a cuticle which lines the anterior and posterior regions of the gut, i.e., the hindgut and the foregut (Chapman, 1998), while the midgut is protected by the peritrophic matrix. This chitinous membrane is present in most but not all the insects and, although permeable to nutrients, it is able to protect the gut epithelium from mechanical damage and invaders (Wigglesworth, 1972). However, if pathogens overcome these barriers, they rapidly spread in the insect hemolymph. The open circulatory system of insects facilitates a rapid dissemination of *non-self* agents but, on the other hand, it also allows a rapid diffusion of the effectors and mediators of the immune system. The insect must trigger a rapid immune response that interrupts the life cycle of the invaders to survive. While vertebrates have developed two defense mechanisms (i.e., innate and acquired immunity), insect immunity shows no

immunoglobulin-based memory, but involves rapid cellular and humoral responses (innate immunity) (Müller et al., 2008).

Recognition of non-self agents

Insects are able to discriminate between *self* and *non-self* agents through a highly conserved mechanism that involves the detection and interaction of Pattern Recognition Receptors (PRRs) with molecules present on the surface of the invader (Buchon et al., 2014). These are collectively named Pathogen-Associated Molecular Patterns (PAMPs) and include peptidoglycans, lypopolysaccharides, β-1,3 glucans, and other sugars which are important for the survival of invading microorganisms (Theopold et al., 1999; Janeway and Medzhitov, 2002). PRRs have been well characterized and can be grouped in peptidoglycan recognition proteins (PGRPs), β- 1,3- glucan recognition proteins (βGRP), C type lectins, and hemolin (Rosales, 2017).

PGRPs are proteins conserved from invertebrates to vertebrates, and have been identified in insects, mollusks, echinoderms, fish, birds, and mammals (Dziarski, 2004). In insects, they have been characterized by Yoshida and colleagues (1996) in the silkworm, *Bombyx mori*. Up to 19 PGRPs are present in insects and they can be classified in short and long form subfamilies: the first are present in the hemolymph, cuticle, and expressed in fat body, while the second are expressed in the hemocytes (Charroux et al., 2009; Royet et al., 2011). In Diptera, and more precisely in *Drosophila melanogaster*, 12 genes coding for PGRPs have been identified, but it must be underlined that not all of them have a function in the activation of the immune system in the presence of *non-self* (Werner et al., 2000; Mellroth et al., 2003). These proteins have a common PGRP domain, which is evolutionarily related to bacteriophage type II amidases and, on the basis of the maintenance or loss of their enzymatic activity, they can be classified in catalytic

or recognition PGRPs (Werner et al., 2000). Four main functions of PGRPs in the immune response of *D. melanogaster* are known: 1) activation of a serine protease cascade that triggers the phenoloxidase system, 2) production of antimicrobial peptides through the *Imd* and *Toll* pathways (Takehana et al., 2002; Royet, 2004), 3) activation of phagocytosis, and 4) removal of peptidoglycans in the hemolymph (Rämet et al., 2002; Mellroth et al., 2003).

As suggested by their name, the plasma family of βGRP recognize and bind to β-1,3-glucans that are present on the cell wall of Gram-negative bacteria. As a consequence, the proPO cascade is activated and, in *Drosophila*, the production of some antimicrobial peptides, as cecropin, drosocyn and attacin, occurs (Wang et al, 2011; Kim et al., 2000).

C-type lectins (CTLs) and hemolin appear to be unique of Lepidoptera, as to date they have not been found in other insect orders (Jiang et al., 2010). Both are able to bind to bacterial lipopolysaccharides (LPS) and lipoteichoic acid (LTA), but while CTLs induce agglutination of bacteria and yeast, helping hemocytes to eliminate pathogens, hemolin induce phagocytosis and nodulation (Yu et al., 2002; Eleftherianos et al., 2007; Yu et al., 2006).

The innate immune system of insects

As mentioned above, the immune system of insects includes humoral and cellular responses (Fig 1). Humoral responses involve effectors that are soluble molecules, such as Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), antimicrobial peptides (AMPs), lysozyme, and the enzymatic cascade that allows hemolymph clotting and production of melanin (Hoffman, 1995; Siva-Jothy et al., 2005). Conversely, cellular defense refers to processes as

phagocytosis, nodulation, and encapsulation mediated by hemocytes (Gillespie et al., 1997; Lavine and Strand, 2002).

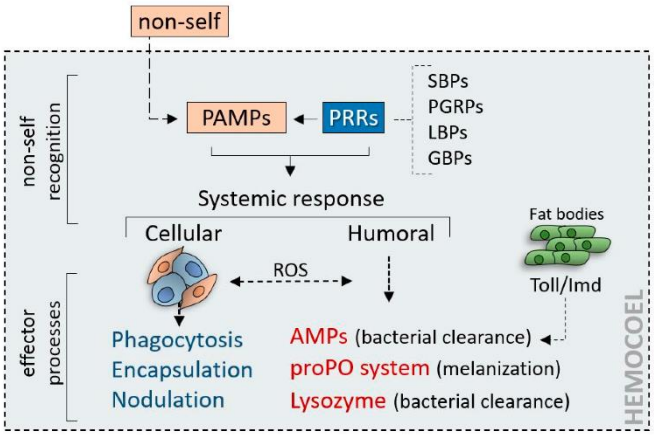

Figure 1. The immune system of insects (modified from Brivio and Mastore, 2020).

Although these two components have always been described as two distinct elements of the immune system, increasing evidence on a cross-talk between them is available. The recognition of the extracellular stimulus leads to the activation of a network of different pathways of signal transduction that allow to elicit the most adequate response to cope with the pathogen (García-Lara et al., 2005). The fat body and the hemocytes, which are the main actors of the immunity, activate the production of humoral factors and cellular response that cooperate each other to stop the invaders (Tsakas and Marmaras, 2010). For example, different papers report the link between the melanization and phagocytosis processes, corroborating this hypothesis (Elrod-Erickson et al., 2000; Hillyer et al., 2003; Mavrouli et al., 2005; Falabella et al., 2012; Di Lelio et al., 2014).

The cellular response

Hemocytes and hematopoiesis

Hemocytes are cells which can freely circulate in the hemocoel or be attached to various organs and tissues (King and Hillyer, 2013). Over the years the nomenclature of insect cells raised a lot of debates, and even the identification of hemocyte types in different insect species is controversial (Price and Ratcliffe, 1974; Brehélin and Zachary, 1986). Historically, hemocytes have been classified according to their morphological, functional, and histochemical features (Brehélin and Zachary, 1986).

Hemocytes in Diptera

In Diptera, the most detailed description of hemocytes concerns *Drosophila* larval blood cells, which are named plasmatocytes, crystal cells, and lamellocytes (Lanot et al., 2001; Wertheim et al., 2005) (Fig 2).

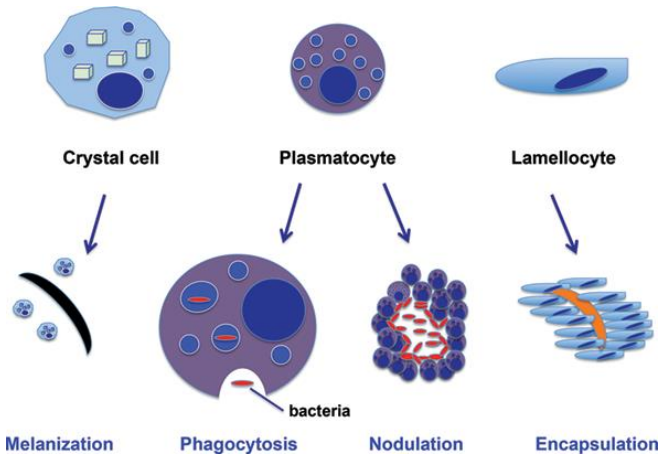

Figure 2. *Drosophila* hemocytes: types and functions (Rosales, 2017).

The precursors of these cells are named prohemocytes and principally reside in hematopoietic organs (Jung et al., 2005). They are very small, round or oval cells

(4 to 8 μm) with a large nucleus and poor in organelles (Chapman, 1998; Silva et al., 2002; Giulianini et al., 2003). They represent only 1% to 7% of the hemocyte population (Brehélin and Zachary, 1986; Chapman,1998).

Plasmatocytes are the most representative cells in the hemolymph of the larva (90-95% of mature hemocytes) (Strand, 2008). They are typically spherical, small cells (around 9/10 μm in diameter), with a central nucleus, a moderately basophilic cytoplasm and large lamellipodial protrusions (Honti et al., 2014). The main function of plasmatocytes is the engulfment of pathogens and other small particles, but they could also be implicated in the activation of AMP production (Honti et al., 2014; Brennan et al., 2007). In fact, pathogen recognition leads plasmatocytes to secrete Psidin, which is considered a signaling factor between these cells and the fat body (the main organ responsible for AMP synthesis) (Brennan et al., 2007). Furthermore, it has been demonstrated that these hemocytes are involved in the formation of capsules that are necessary for the encapsulation of large invaders. During this process, plasmatocytes strictly adhere each other by tight junctions and form a homogeneous layer around the *non-self* agent, which is used as a basis for the subsequent adhesion of lamellocytes (Williams et al., 2005).

The non-adhesive crystal cells comprise less than 5% of the hemocyte population and are distinguishable for the presence of crystal inclusions in the cytoplasm (Honti et al., 2014). They express prophenoloxidase 1 (PP1), a fundamental component for the activation of the phenoloxidase cascade, which culminates with the production of melanin during wound healing or capsule formation (Strand, 2008).

Finally, lamellocytes are flat and large (40 μm in diameter) cells, usually absent in healthy larvae. They can rapidly differentiate from prohemocytes following the activation of the immune response and are involved in the encapsulation of

large targets, in cooperation with plasmatocytes and crystal cells (Lanot et al., 2001; Honti et al., 2014).

In other insect orders (e.g., Diptera other than *Drosophila*, Lepidoptera, Coleoptera, Hymenoptera, and Hemiptera) circulating and differentiated cells are usually classified differently from *Drosophila* and they are named plasmatocytes, granulocytes, oenocytoids, and spherule cells (Lavine and Strand, 2002; Ribeiro and Brehélin, 2006; Hillyer and Strand, 2014).

Hemocytes in Lepidoptera

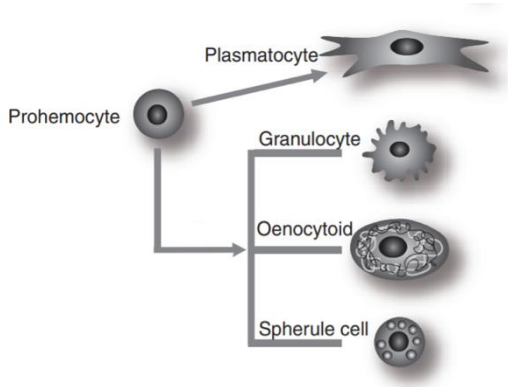

Figure 3. Hemocyte types in Lepidoptera (modified from Strand, 2008).

In Figure 3 the main hemocyte types of Lepidoptera are outlined. As in *Drosophila*, plasmatocytes are among the most numerous hemocytes (Chapman, 1998). Plasmatocytes are polymorphic cells, round or oval while they are free in the hemolymph, but they become flat and spread with filopodia when adhere on a surface. Their nucleus is often central and the cytoplasm contains several organelles, as a moderate amount of rough endoplasmic reticulum (RER), a poorly developed Golgi apparatus and, in *Galleria mellonella*, some granules (Ribeiro and Brehélin, 2006). As in Diptera, they play an important role in phagocytosis, nodule formation, and encapsulation of *non-self* agents (Lavine and Strand, 2002).

The most abundant cells are granulocytes and, together with plasmatocytes, they represent the most abundant circulating cells (over the 30% of the total cell count). They are considered "professional phagocytes" (Strand et al., 2006). In

fact, their main role in phagocytosis has been demonstrated in several species of Lepidoptera as *B. mori*, *G. mellonella*, and *Spodoptera littoralis* (see Ribeiro and Brehélin, 2006). Moreover, they appear to be the first cells that come into contact with pathogens during nodule formation (Ribeiro and Brehélin, 2006). They can be distinguished by their granular content and are characterized by several lysosomes, free ribosomes, and a developed RER (Chapman, 1998; Ribeiro and Brehélin, 2006).

Oenocytoids are very large cells (from 16 to 54 μm), with an eccentric nucleus and a homogenous or inhomogeneous cytoplasm depending on the species (Ribeiro and Brehélin, 2006). They are structurally and functionally similar to *Drosophila* crystal cells, in fact they contain the pro-phenoloxidase system (pro-PO) (Ribeiro et al., 1996).

Spherulocytes are rounded cells characterized by the presence of inclusions, named spherules, in the cytoplasm with granular content. The functions of these cells remain unclear, although some authors hypothesized that they are involved in melanization and phagocytosis (Chapman, 1998; Ribeiro and Brehélin, 2006).

Finally, similarly to *Drosophila*, prohemocytes represent undifferentiated, progenitor cells (Strand, 2008).

Hematopoiesis

Hemocytes are produced in two different phases of the insect development (Holz et al., 2003). The head or the dorsal mesoderm produce the first population of hemocytes during embryogenesis while successively, during larval and nymphal stages, the second population is produced by hemopoietic organs. In particular, in *D. melanogaster* these organs are called lymph glands, which are formed by paired primary and secondary lobes along the anterior end of the dorsal vessel (Shrestha and Gateff, 1982). While secondary lobes mostly contain

prohemocytes, primary lobes can be divided in a 1) cortical zone with plasmatocytes and crystal cells, 2) a medullary zone, which contains quiescent prohemocytes, and a 3) posterior signaling center (PSC) that contains putative prohemocytes (Jung et al., 2005). During the larval stage of the fruit fly, hemocytes derive from the division of circulating cells, as well as from the production of new cells by hematopoietic organs (Holz et al., 2003). Similarly to Diptera, hematopoietic organs in Lepidoptera are involved in the production of plasmatocytes mostly during larval development. Conversely, the other types of hemocytes (i.e., granulocytes, oenocytoids, and spherule cells) derive from cells that are already circulating in the hemolymph (Strand, 2008).

Phagocytosis

Phagocytosis is a widely conserved process among the animal kingdom and probably represents the oldest defense response against pathogens. The meaning of phagocytosis as "internal defense process" was introduced in 1880s and since that moment it has been widely studied.

Through phagocytosis cells recognize, bind, internalize, and destroy small particles. This process can begin either following the direct recognition of the pathogens through specific receptors present on the surface of the hemocytes, or following the binding of opsonin ligands to the outer surface of pathogens, increasing their recognition by the receptors (Browne et al., 2013; Hillyer, 2016).

Differently from Lepidoptera, in which different kinds of opsonins are able to bind both Gram-positive and Gram-negative bacteria, and also yeasts (Li et al., 2014; Zhan et al., 2016; Di Lelio et al., 2019), thioester-containing proteins (TEP) are the main actors involved in the opsonization and phagocytosis of pathogens in Diptera (Stroschein-Stevenson et al., 2006).

After the recognition of the invaders by cell surface receptors, signaling pathways are activated, leading the cell to adhere to the invader surface through marked changes in their plasma membrane and cytoskeleton (Rosales, 2011). Pseudopods are extended around the particle and the plasma membrane closes in a few minutes the distal end forming a phagosome (Yeung et al., 2006). The following step is "phagosome maturation" during which the membrane of the phagosome fuses with other organelles (e.g., lysosomes and endosomes) resulting in the formation of an acid compartment in which hydrolytic enzymes and lysozymes cause the destruction of the target (Yeung et al., 2006). In insects, and more precisely in *G. mellonella*, Metalnikov and Chorine (1929) firstly demonstrated the role of hemocytes in this process, and since then several papers on this topic have been published. As mentioned before, phagocytosis in Diptera is triggered by plasmatocytes, although the involvement of different cell types in different insect species cannot be excluded (Lavine and Strand, 2002; Rosales, 2011). Through phagocytosis, animals are able to eliminate both microorganisms, thus controlling a bacterial infection, and apoptotic cells (Rosales, 2011). This last aspect is very important during embryogenesis and metamorphosis of holometabolous insects as *Drosophila* when obsolete or unnecessary cells undergo apoptosis and need to be removed from the insect body (Neufeld and Baehrecke, 2008).

Encapsulation

Hemocytes are also involved in another defense mechanism by which biotic (e.g., nematodes, protozoa, and parasitoids) and abiotic (e.g., nylon threads or plastic beads) agents of larger dimensions are isolated in a capsule-like envelope (Wigglesworth, 1979; Götz, 1986). "Cellular encapsulation" has been demonstrated more or less in all insect groups that have been investigated so far.

Encapsulation begins with the recognition of the foreign invader: this is driven by an initial, random contact between hemocytes and the invader. The activation of a signaling pathway leads to cell adhesion, spreading, and degranulation (Dubovsky et al., 2016). In Lepidoptera, granulocytes are the first cells that contact the foreign target. Once degranulated, they release chemotactic components that attract plasmatocytes (Lavine and Strand, 2002). These attach to granulocytes forming a multilayered capsule around the invader, which is killed by the release of cytotoxic products (as ROS or melanin), asphyxia or starvation (Nappi et al., 1995; Carton et al., 2009). The same process is carried out by lamellocytes and crystal cells in *Drosophila* (Rosales, 2017). The aggregation of hemocytes in multiple layers around the target seems to involve integrins, both in Diptera and Lepidoptera (Irving et al., 2005; Zhuang et al., 2008)

A second type of encapsulation (called "humoral encapsulation") has been described in Diptera, which involves the deposition of melanin around the foreign target (usually nematodes, fungi, and bacteria) without the formation of a cell capsule (Gotz and Vey, 1986). In this case, a soft and colorless capsule containing the activated phenoloxydase enzyme is formed around the invader. Then it solidifies and changes the color to dark brown, thus isolating the invader (Gotz and Vey, 1986).

Nodule formation

The process of nodulation is more documented in Lepidoptera, but it has also been described in other orders as Diptera, Orthoptera, and Hemiptera (see Satyavathi et al., 2014). It is triggered by the presence of a huge amount of foreign microorganisms that hemocytes cannot phagocytize thus, thanks to the formation of multicellular aggregates, a large number of pathogens can be entrapped (Rosales et al., 2017). This process is very effective against bacteria,

fungi, yeast, and some protozoans (Garcia et al., 2012; Satyavathi et al., 2014). Upon the recognition of the invaders, hemocytes change their morphology and behavior from circulating cells to adherent cells. However, the molecular mechanisms behind these changes have not been clearly defined yet (Lavine and Strand, 2002; Satyavathi et al., 2014). Although granulocytes and plasmatocytes are the only cells involved in nodule formation, oenocytoids could have a possible indirect role at the end of the process for the production of melanin (Gillespie et al., 1997; Chapman, 1998). Conversely, capsules are mostly formed by lamellocytes in *Drosophila*, but how these cells are recruited, and if other cell types are involved, is unclear (Strand, 2008).

The humoral response

The recognition of the *non-self* target also leads to the activation of a humoral response, which includes wound healing in response to injury, hemolymph clotting, melanization, and the production of humoral factors (e.g., antimicrobial peptides, lysozyme, and lectins).

Antimicrobial peptides (AMPs)

Antimicrobial peptides are small peptides (generally between 12 and 50 amino acids), which can rapidly respond to pathogen infections (Yi et al., 2014). These proteins are mainly produced by fat body cells, although it seems that they can also be synthesized by hemocytes and epithelial cells in the intestinal tract and the epidermis (Schmid-Hempel, 2005; Lemaitre and Hoffmann, 2007).

Insects produce a large variety of AMPs and, according to their amino acid sequence and structure, they can be classified into 1) α-helix linear peptides, without cysteine residues, 2) cysteine-rich peptides, 3) glycine-rich proteins, and 4) proline-rich peptides (Bulet and Stöcklin 2005; Otvos, 2000). Linear AMPs have a length of about 29-42 residues and they do not present cysteine residues. Due

to this lack, they are unstructured in an aqueous environment, but they fold and assume an α-helix conformation in a hydrophobic environment (Bulet and Stöcklin, 2005; Lewies et al., 2015). Cecropins, the most abundant AMPs, and moricins, which were identified only in Lepidoptera, belong to this group (Yi et al., 2014). Cecropins were isolated in 1980s in the cecropia moth, *Hyalophora cecropia* (Steiner et al., 1981) and, since that time, they have been well characterized both in lepidopteran and dipteran species (Wu et al., 2018). They act against both Gram-positive and Gram-negative bacteria, lysing the bacterial membrane or causing leaks in their membrane (Wu et al., 2018). Moreover, some studies demonstrated that Cecroprins also have antifungal properties (Ekengren and Hultmark, 1999; Lowenberger et al., 1999). The N-terminus of cecropins shows a long, polarized amphipathic helix, and is linked by a glycine-proline region to the hydrophobic C-terminus. Moreover, in most insects there is a tryptophan residue in position 1 or 2 (Bulet and Stöcklin, 2005). Tryptophan is present for example in cecropin of *D. melanogaster*, while the lack of this amino acid was reported in *Anopheles gambiae*, *Aedes aegypti* and also in cecropins of *B. mori* (Lowenberger et al., 1999; Vizioli et al., 2000; Hara et al., 1994). It should be noted that in *A. gambiae* the absence of tryptophan is associated with a greater efficiency in contrasting Gram-positive bacteria (Vizioli et al., 2000). A structure of an insect cecropin is reported in Fig 4a.

The second class of cysteine-rich AMPs includes defensins and drosomycins (Yi et al., 2014). In particular, defensins were initially isolated from humans and plants and so named according to their similarity to mammalian counterparts (Bulet and Stöclin, 2005; Yi et al., 2014; Zhao et al., 2015). They are widely present among insect orders, including Hemiptera, Odonata, Coleoptera, Diptera, and Lepidoptera (Mylonakis et al., 2016; Wu et al., 2018). They consist of 29-34 amino acids and show 6-8 conserved cysteine residues. They are composed by an α-

helix and an antiparallel β-sheet, linked together by two disulfide bridges, which can form a motif named "cysteine-stabilized alpha beta (CSαβ)", typical also of *D. melanogaster* drosomycin, or a "loop-helix-beta-sheet" structure (Cornet et al., 1995; Landon et al., 1997) (Fig 4b,c). Defensins are highly active against Gram-positive bacteria (as *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*) (Wu et al., 2018), but some are also active against *E. coli* (Gram-negative bacteria) and fungi (Vizioli et al., 2001; Seufi et al., 2011). Finally, the inducible AMP drosomycin, isolated only from *D. melanogaster*, has very high antifungal properties, while no activity against yeast and bacteria was observed (Bulet et al., 1999; Imler and Bulet; 2005). It contains eight cysteines that are involved in the formation of four disulfide bridges inside the molecule, which confer compactness to the protein structure (Michaut et al., 1996).

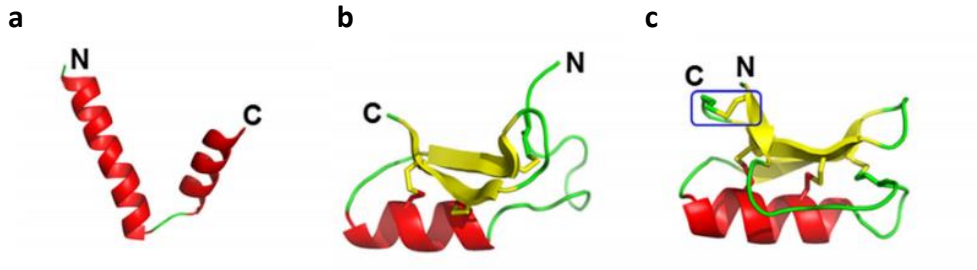

Figure 4. Structure of insect AMPs: cecropins (a), defensins (b), and drosomycins (c) (modified from Yi et al., 2014).

No cysteine residues are present in glycine-rich and proline-rich AMPs and any particular and regular secondary structure have been observed (Lewies et al., 2015). Glycine-rich AMPs include different polypeptides isolated from different insect orders. Among these, diptericins and sarcotoxins (Diptera), attacins (Diptera and Lepidoptera), gloverins (Lepidoptera), and other AMPs typical of Hymenoptera, Coleoptera and Hemiptera (Bulet et al., 1999). As concern diptericin, this AMP was isolated by the northern blowfly, *Phormia terranovae*,

but it is also produced in *D. melanogaster* (Dimarcq et al., 1988; Reichhart et al., 1992). This protein consists of a glycine-rich domain in the C-terminal part and a short proline-rich domain in the N-terminus (Hedengren et al., 2000). Diptericins and attacins are very similar in both their structure and activity against Gram-negative bacteria (Hedengren et al., 2000). Finally, apidaecins and abaecins (from Hymenoptera), drosocin and metchnikowin (from Diptera), pyrrhocoricin (from Hemiptera) and lebocins (from Lepidoptera) (see Bulet et al., 1999) belong to the proline-rich group.

AMPs are known mostly for causing the disruption of the plasma membrane of the invading microorganisms, although the mechanisms by which AMPs kill bacteria are different. First of all, the contact between these peptides and the target strongly depends on the lipid composition of the bacterial membrane and occurs through a hydrophobic or electrostatic interaction (Yeaman and Yount, 2003).

Four models of AMPs mode of actions have been described to date (Melo et al., 2009) (Fig 5):

1) In the "toroidal model", AMPs aggregate and insert perpendicularly in the membrane bilayer, inducing a membrane curvature. The lumen of the pore is lined both by AMPs and phospholipid head groups, generating a continuity between the outer and inner membrane sheets (Ludtke et al., 1996)
2) A variation on the theme, named "disordered toroidal-pore", has been proposed by Leontiadou and colleagues (2006), in which the orientation of peptides is not ordered and the pore is not totally cylindrical.

3) In the "barrel-stave model" the peptides insert perpendicularly in the bilayer, producing a pore in which the lumen is formed only by AMPs (Baumann and Mueller, 1974).
4) In the "carpet model", peptides accumulate on the membrane and, when the AMP concentration is high, they produce a "detergent-like" effect and disintegrate the membrane (Oren et al., 1998).

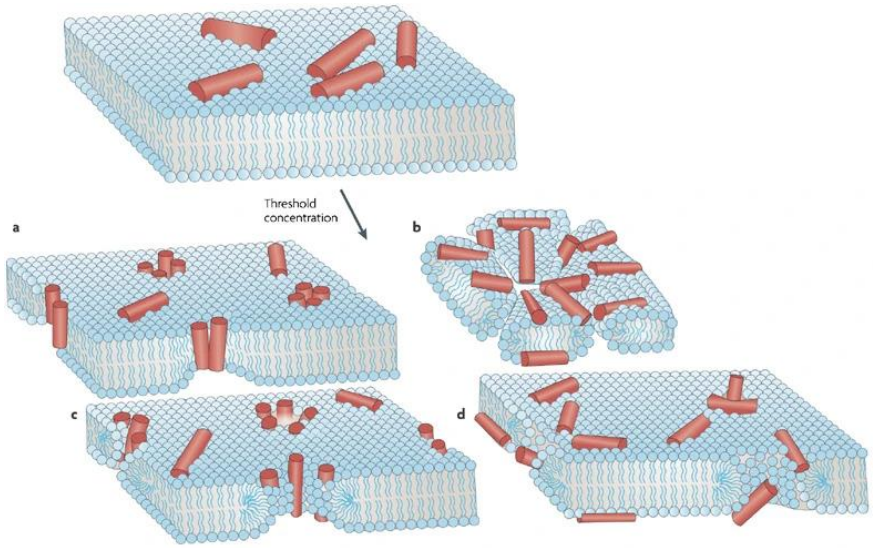

Figure 5. Mode of action of antimicrobial peptides. a) Barrel-stave pore model; b) Carpet mechanism; c) Toroidal pore model; d) Disordered toroidal pore model (Melo et al., 2009).

More recently, increasing evidence is available on how some AMPs can also act on intracellular targets (e.g., proteins and nucleic acids), after their direct translocation to the cytoplasm of the cell or following endocytosis (Hale and Hancock, 2007; Madani et al., 2011; Le et al., 2017). To date, little is known about insects and the majority of information regards protein-folding inhibitors (see Le et al., 2017). An example is *Drosophila* drosocin which, after a first interaction with the bacterial lipopolysaccharides (LPS), inactivates chaperones involved in protein folding, resulting in the death of the pathogen (Otvos et al., 2000).

Prophenoloxidase (proPO) system

Melanogenesis is very important in the response against pathogens and for repairing tissues. It leads to the production of pigment-forming chemical compounds, named melanins, which are present in animals, plants, and protists (Nappi and Christensen, 2005; Gonzáles-Santoyo and Córdoba-Aguilar, 2012). In invertebrates, the main actor that drives the activation of melanogenesis is the enzyme phenoloxidase (PO) (Wyatt, 1961), which is mainly synthetized in hemocytes as an inactive zymogen of 70-80 kDa, i.e. prophenoloxidase (proPO) (Nappi and Christensen, 2005).

Melanization starts with the recognition of a *non-self* target by PRRs localized on the hemocytes. It has been demonstrated that, in many insect species, β-1,3-glucan, lipopolysaccharides, and peptidoglycan are the most effective stimulators of this process (Strand, 2008; Gonzáles-Santoyo and Córdoba-Aguilar, 2012). As a consequence, proPO is activated through a proteolytic cleavage by the proPO-activating enzyme (PPAE), and subsequently PO catalyzes the formation of melanin through the *o*-hydroxylation of monophenols and the oxidation of phenols to quinones (Nappi and Christensen, 2005; Gonzáles-Santoyo and Córdoba-Aguilar, 2012). Briefly, at the begging of the process, PO hydroxylates tyrosine forming L-3-4-dihydroxyphenilalanine (L-DOPA), which is oxidized producing dopaquinone that is subsequently converted to dopachrome (Nappi and Christensen, 2005). This is converted to 5,6-dihydroxyindole (DHI) by dopachrome conversion enzyme. DHI is oxidizes by PO in indole-5,6-quinone, which is finally cross-linked with hemolymph proteins to form melanotic capsules. Moreover, a complementary pathway exists, in which dopa decarboxylase hydroxylates L-dopa, leading to the formation of dopamine, which is converted to melanin by PO and other enzymes

(Nappi and Christensen, 2005). The enzymatic and non-enzymatic reactions which lead to melanin formation are shown in Fig 6.

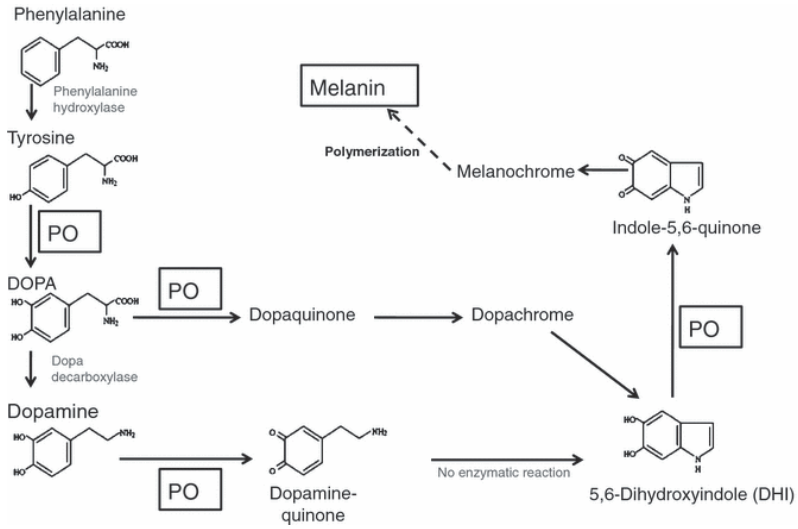

Figure 6. Melanin synthesis (González-Santoyo and Córdoba-Aguila, 2012).

Once synthetized, melanin is deposited within nodules formed by aggregated hemocytes, which impair the growth of the pathogen, and mostly the absorption of nutrients, leading to its death (Gillespie et al., 1997). Moreover, melanin deposition frequently occurs in the encapsulation response to parasites or after the injection of foreign objects in the insect body (such as sepharose beads) (Kanost and Gorman, 2008).

During melanin synthesis, several molecules such as proteases, cytotoxic quinines, nitrogen intermediates, and cytotoxic reactive oxygen species are produced, which are also implicated in eliminating pathogens (Nappi and Christensen, 2005). However, these molecules can damage the host itself if produced in excess and, therefore, regulated production of melanin is necessary (Nappi and Christensen, 2005). In particular, the response may be localized and limited in time. To this purpose, different studies described the presence of serine

protease inhibitors in the insect hemolymph (Kanost, 1999). The best studied group of serine protease inhibitors are serpins, a family of 50 kDa proteins that act on PPAEs (Gettins, 2002). There are different lines of evidence on the role of serpins in the regulation of the immune response of insects. In particular, their role during the melanization process has been described in *D. melanogaster*, in *Tenebrio molitor*, in *A. aegypti* and *A. gambiae* (Michel et al., 2006; Ahmad et al., 2009; Jiang et al., 2009; Zou et al., 2010). However, in most of these cases the proteinases, which are inhibited by serpins, have not been identified yet. An exception is *Manduca sexta*, in which the target for three of the seven serpins identified has been retrived: Serpin-1 splicing isoform J (serpin-1J) and Serpin-3 regulates proPO activation by inhibiting PPAEs, while Serpin-6 by inhibiting PPAE3 (Zhu et al., 2003; Gupta et al., 2005; Zou and Jiang, 2005). In addition to serpins, an efficient 4 kDa PO inhibitor peptide was found in *Musca domestica* hemolymph, which directly inhibits PO rather than the activating proteases (Tsukamoto et al., 1992).

Lysozymes

The innate response is also mediated by another important group of enzymes named lysozymes, which are able to cleave the glycosidic bond between *N*-acetylmuramic (NAM) and *N*-acetylglucosamine (NAG) residues of bacterial peptidoglycan. The first lysozyme was discovered in 1922 by Fleming and since then homologs have been characterized in a variety of organisms (Yu et al., 2002). Focusing on the animal kingdom, these enzymes can be classified into:

1. c-type (chicken or conventional type) lysozymes are mostly produced by vertebrates, but have also been found in several arthropods (e.g., crustaceans, arachnids, insects as Lepidoptera, Diptera, and Hemiptera) (Callewaert and Michiels, 2010);

2. g-type (goose-type) lysozymes were initially discovered in vertebrates, but recently they have been also identified in some invertebrates. However they are absent in insects and in nematodes (Callewaert and Michiels, 2010);
3. i-type (invertebrate type) lysozymes: these enzymes are typical of invertebrates. Studies on the genome of vertebrates have confirmed the total absence of genes coding for i-type lysozymes in this *taxon* (Callewaert and Michiels, 2010).

In insects, different isoforms of lysozymes have been discovered in Diptera, Lepidoptera, Coleoptera, and Orthoptera (Hernandez et al., 2003; Adamo, 2004; Gorman et al., 2004; Gandhe et al., 2006). The fat body is the main organ where lysozyme is produced, although circulating hemocytes also represent an important additional site of the production of this enzyme (Dunn et al., 1985). Moreover, in Diptera as *D. melanogaster*, *M. domestica*, and *H. illucens* lysozymes are acidic proteins also present in midgut cells (Kylsten et al., 1992; Padilha et al., 2009; Bonelli et al., 2019). In this region, lysozymes may play an important role in promoting the elimination of the pathogens ingested with food. The production of lysozyme in midgut cells seems to be an adaptation to the saprophytism of the larvae of these insects in which the intestinal pH creates an optimal environment for its activity (Hultmark, 1996). Unlike AMPs, lyzozymes are constitutively expressed both in the gut and in the hemolymph, but in this compartment their production increases following the entrance of pathogens (Dunn et al., 1985). The constitutive expression of lysozyme could be involved in the modulation of the immune response: it seems that these proteins start to perform their defense function even before the recognition of the pathogen, promoting the breakdown of small components of the cell wall, thus stimulating

plasmatocytes to phagocyte invading microorganisms (Dunn et al., 1985; Park et al., 2007).

Model organisms

The silkworm, *Bombyx mori*

The experimental model used for the first part of this work is the silkworm *B. mori*, which belongs to the order of Lepidoptera. The growth and survival of silkworm completely depend on humans and for this reason it is considered a domesticated animal (Goldsmith et al., 2005).

As a holometabolous insect, the life cycle of *B. mori* involves four stages: egg, larva, pupa, and adult (or imago) (Fig 7).

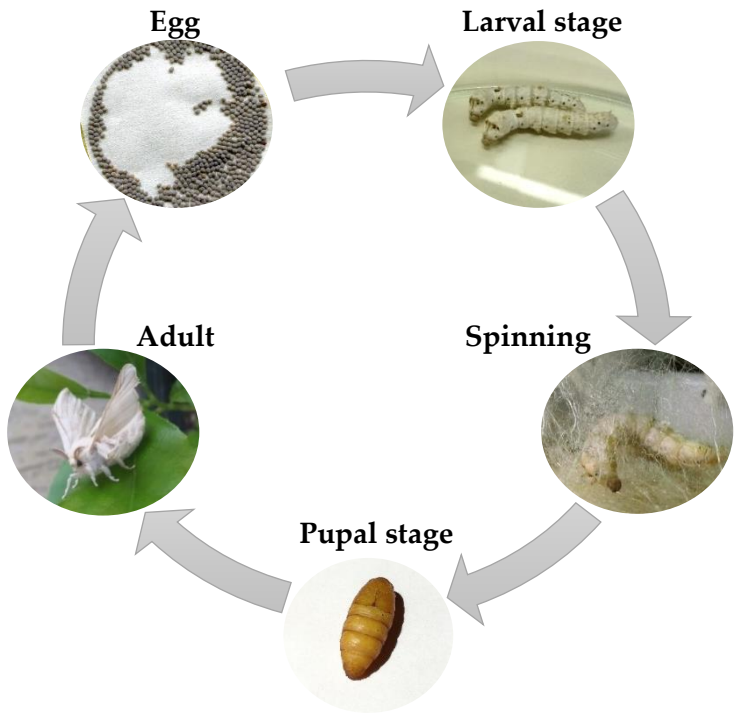

Figure 7. Life cycle of *B. mori* (images provided by CREAA-API, Centro di Ricerca Agricoltura e Ambiente, Padova, and Aurora Montali, Università dell'Insubria, Varese).

After hatching, the larva actively feeds on mulberry leaves. It goes through five instars, which are separated by molts, and at the end of the last larval instar the silkworm stops feeding and starts to spin the silk cocoon (spinning). At pupal stage, the insect undergoes metamorphosis and, after about ten days, the adult emerges.

As a natural producer of silk, this insect has a fundamental role in sericulture. In addition to its use in the textile industry and clothing production, silk has recently gained attention as biomaterial thanks to its peculiar properties, as biocompatibility and biodegradability (Reddy and Prasad, 2011). Moreover, it can be used in the cosmetic and pharmaceutical sectors thanks to the antioxidant properties and biocompatibility of sericin, one of the component of the silk thread (Wu et al., 2007).

Besides its economic importance, *B. mori* is of great interest for basic research and has progressively gained attention in insect biotechnology (Goldsmith et al., 2005). It represents in fact a model organism among Lepidoptera since it shows a short generation time, a clear genetic background, and a considerable number of its genes are homologous to human. As a consequence, nowadays a large amount of information on its biology and physiology, as well as different molecular tools to manipulate the larvae are available (e.g., RNAi silencing, gene transfer, and CRISPR/Cas9 gene editing), and the genome is completely sequenced (Wang et al., 2005; Meng et al, 2017). More recently *B. mori* has also proved to be an interesting alternative to the use of mammals in preclinical studies for the screening of new antimicrobial drugs, and to study bacterial infections (Kaito et al., 2002; García-Lara et al., 2005; Sekimizu et al, 2012). Silkworm models for studying bacterial and fungal infections are under development and the availability of this faster and cheaper system could boost the selection of potential compounds in the initial phase of research and

development of new antibacterial and antifungal molecules (Kaito and Sekimizu, 2007).

The Black Soldier Fly, *Hermetia illucens*

H. illucens, also known as Black Soldier Fly (BSF), has been used in the second part of this PhD project. This insect belongs to the order of Diptera and, nowadays, it is a non-pest insect worldwide distributed (Marshall et al., 2015).
A schematic life cycle of *H. illucens* is reported in Fig 8. After mating, the female fly lays about 500-900 eggs, which hatch approximately in four days. The larval phase of *H. illucens* is divided into six different instars, and this phase can last from two weeks to four months in relation to trophic and environmental conditions (Hardouin and Mahoux, 2003). During the larval period, the insect is able to grow on the most different kinds of substrates, including for example fruits and vegetables in decomposition, waste from the agri-food supply chain, and even manure (Diener et al., 2011; Zhou et al., 2013; Liland et al., 2017; Beskin et al., 2018; Xiao et al., 2018). Once the larval phase is concluded, the insect transits through a prepupal and then a pupal stage, which has a duration of about two weeks but can even last five months under unfavorable environmental conditions (Hardouin and Mahoux, 2003; Tomberlin, 2009). Despite the survival of the adult insect seems to depend only on the reserves accumulated in the fat body during the larval stage and the fly does not require an additional trophic supply (Diclaro and Kaufman, 2009; Salomone et al. 2016), recent studies have shown that the adult lifespan significantly increases in the presence of water and food sources (Nakamura et al., 2016; Bertinetti et al., 2019; Bruno et al., 2019b).

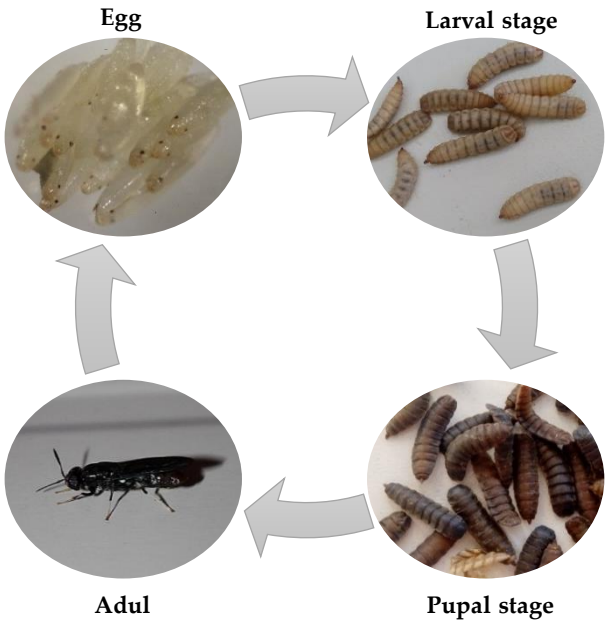

Figure 8. Life cycle of *H. illucens* (images by Daniele Bruno and Aurora Montali, Università dell'Insubria, Varese).

Currently, researchers are very interested in studying BSF larvae and this stems from different reasons. For example, this insect is very used in forensic entomology for estimating human postmortem interval (Turchetto et al., 2001). Furthermore, it has been used for biotechnological studies, since it is a source of natural compounds with medical potential (i.e., chitin and antimicrobial peptides, respectively) (Vogel et al, 2018; Liu et al., 2019). Among all, the greatest potential of this saprophagous insect is its ability to convert organic waste. As mentioned before, BSF can be reared on several substrates and besides to the reduction of organic matter, these insects are able to accumulate fat and proteins of high nutritional quality, thus representing an important resource that can be used for the production of chicken and fish feed (Newton et al., 2005; Makkar et al., 2014). The value of the final product depends on the feeding substrate on which larvae are reared, as well as the rearing conditions (Wang and Shelomi,

2017). About this aspect, to avoid possible contamination the European commission set up a list of the substrates that are allowed: for example former foodstuffs not containing meat and fish are currently used (EFSA Scientific Committee, 2015; European Commission, 2017). In addition to waste bioconvertion, this insect is able to reduce the bacterial load (e.g., *E. coli* and *S. enterica*) and the total amount of microelements (e.g., nitrogen or phosphorous) and heavy metals (e.g., copper, iron, nickel, lead, chromium) in the substrate, thus reducing also its pollution potential (Erickson et al., 2004; Liu et al., 2008; Sheppard et al., 2008).

The final aim is thus to create a circular economy system based on the mass rearing of *H. illucens*. The feeding habits of the larvae put them continually in contact with a broad spectrum of microorganisms (including pathogens), and so the evaluation of biological safety aspects related to the use of this insect is extremely important (Čičkova et al., 2015; Nguyen et al., 2015; Zdybicka-Barabas et al., 2017). Therefore, the characterization of the insect immune system is mandatory. This can allow to understand how the larvae of this Diptera are able to respond to infections getting during the bioconversion process, improving the health of the animals and avoiding a potential introduction of pathogens in the production chain. As mentioned above, this insect can be considered a source of AMPs, which can be used as additives in poultry and swine production, leading to different positive effects, for example, on the digestibility or on the gut health (Wang et al., 2016).

The future manipulation of the immune system of *H. illucens* could offer a real possibility to improve the value of insect-derived products as well as to increase the insect resistance to infections, to set up methods and protocols useful for increasing AMP production thus exploiting insects to isolate these molecules, which can be used for different purposes.

AIM OF THE PROJECT AND STRUCTURE OF THE THESIS

The projects that have been developed during this PhD course are reported in the following two chapters. More in detail:

CHAPTER 1 reports the development of an insect infection model. In particular, *B. mori* larvae infected with *S. aureus* were used to compare the efficacy of three glycopeptides antibiotics (i.e., vancomycin, teicoplanin, and dalbavancin).

CHAPTER 2 reports the characterization of the immune response of the dipteran *Hermetia illucens* after an immune challenge with a Gram-positive and Gram-negative bacteria mix.

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antibiotics

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Article

A silkworm infection model for in vivo study of glycopeptide antibiotics

Aurora Montali [1,†], Francesca Berini [1,†], Maurizio Francesco Brivio [2,†], Maristella Mastore [2], Alessio Saviane [3], Silvia Cappellozza [3], Flavia Marinelli [1,*] and Gianluca Tettamanti [1,4]

[1] Department of Biotechnology and Life Sciences, University of Insubria, 21100 Varese, Italy; a.montali@uninsubria.it (A.M.); f.berini@uninsubria.it (F.B.); gianluca.tettamanti@uninsubria.it (G.T.).
[2] Department of Theoretical and Applied Sciences, University of Insubria, 21100 Varese, Italy; maurizio.brivio@uninsubria.it (M.F.B.); maristella.mastore@uninsubria.it (M.M.).
[3] Council for Agricultural Research and Economics, Research Centre for Agriculture and Environment (CREA-AA), 35143 Padova, Italy; alessio.saviane@crea.gov.it (A.S.); silvia.cappellozza@crea.gov.it (S.C.).
[4] Interuniversity Center for Studies on Bioinspired Agro-environmental Technology (BAT Center), University of Napoli Federico II, 80055 Portici, Italy
* Correspondence: flavia.marinelli@uninsubria.it; Tel.: +39-033-2421-546
† These authors contributed equally to the work

Received: 27 April 2020; Accepted: 2 June 2020; Published: 4 June 2020

Abstract: Glycopeptide antibiotics (GPAs) are drugs of last resort for treating infections by Gram-positive bacteria. They inhibit bacterial cell wall assembly by binding to the D-Ala-D-Ala terminus of peptidoglycan precursors, leading to cell lysis. Vancomycin and teicoplanin are first generation GPAs, while dalbavancin is one of the few, recently approved, second generation GPAs. In this paper, we developed an in vivo insect model to compare, for the first time, the efficacy of these three GPAs in curing *Staphylococcus aureus* infection. Differently from previous reports, *Bombyx mori* larvae were reared at 37 °C, and the course of

infection was monitored, following not only larval survival, but also bacterial load in the insect body, hemocyte activity, phenoloxidase activity, and antimicrobial peptide expression. We demonstrated that the injection of *S. aureus* into the hemolymph of *B. mori* larvae led to a marked reduction of their survival rate within 24–48 hours. GPAs were not toxic to the larvae and cured *S. aureus* infection. Dalbavancin was more effective than first generation GPAs. Due to its great advantages (i.e., easy and safe handling, low rearing costs, low antibiotic amount needed for the tests, no restrictions imposed by ethical and regulatory issues), this silkworm infection model could be introduced in preclinical phases—prior to the use of mice—accelerating the discovery/development rate of novel GPAs.

Keywords: insect infection model; *Bombyx mori*; glycopeptide antibiotics; vancomycin; teicoplanin; dalbavancin; insect innate immunity

1. Introduction

Over time, the discovery, development, and commercialization of novel antibiotics have dramatically slowed down. Thus, after the 'golden era' of antibiotic discovery that peaked around 1950s, the number of new antibiotics marketed each decade has declined. A recent study indicates that, between 1999 and 2014, only 25 novel antibiotics, belonging to nine chemical classes, were approved worldwide [1]. Conversely, the rapid spread of antibiotic resistance among pathogenic bacteria, considered one of the most alarming threats to global health, makes the development of novel antibacterial drugs compulsory. Notably, ESKAPE pathogens (an acronym used to indicate six multidrug-resistant, nosocomial pathogens, i.e., *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), were recently included by the World Health Organization in the list of bacteria against which new antibiotics are urgently needed [2].

Nowadays, many factors tend to reduce the antibiotic discovery and development success rate, from the difficulty of identifying new essential susceptible bacterial targets, to the regulatory challenges and the limited economic returns that often discourage pharmaceutical companies from investing in the field. Discovering and developing a novel antibacterial may take up to 15 years and 5000 to 10,000 candidates are screened on average, before finding a novel drug that reaches the market [3]. Although clinical trials in humans are the most challenging and expensive development phase, the need to test dozens of drug candidates in animals during preclinical selection represents the major bottleneck along the discovery process [3]. In vivo experiments using mammalian infection models (generally mice and rats) contribute to eliminating toxic compounds, selecting those better curing infections, comparing the efficacy

of administration routes, and optimizing drug formulations [4]. However, the use of mammalian models is expensive and time-consuming. Specific pathogen-free facilities are needed for the growth and maintenance of experimental animals and their use is limited by ethical considerations and regulatory issues. To overcome these problems, alternative invertebrate infection models are desirable [5,6]. Although insects do not have adaptive immune systems, they have evolved innate immunity, which is highly conserved and important for resistance to microbial infections [7]. Different insects, such as *Drosophila melanogaster*, *Galleria mellonella*, and *Bombyx mori*, have recently been used to monitor the course and recovery from infections after antibiotic administration [8–11], and their advantages over mammalian models in terms of ethics, research costs, and speed of experimentation have become more and more evident [12].

In this paper, we investigated the silkworm *B. mori* response to infection of the Gram-positive *S. aureus*, and the subsequent administration of three clinically relevant glycopeptide antibiotics (GPAs). We selected *B. mori* as the infection model, since it is a safe and easy-to-handle insect, with reduced maintenance costs, and does not require special devices for bacterial infection and drug administration, thanks to the big size of the larvae [12]. Antibiotics can be administered to the larvae by several routes and in accurate dosage, and the following isolation of organs and hemolymph does not require particular equipment [13]. Moreover, the occurrence of standard larval instars, the synchronous development of the silkworms during the life cycle, and the reduced inter individual variability improve the reproducibility of the experiments.

GPAs are considered 'drugs of last resort' in treating severe infections by Gram-positive pathogens, such as staphylococci, enterococci, and clostridia [14,15].

Nowadays, multidrug resistant strains of *S. aureus* represent one of the major causes of mortality in hospital-acquired infections [16,17]. The GPAs tested in this study are the first-generation vancomycin and teicoplanin, natural molecules produced by filamentous actinobacteria, and the second-generation dalbavancin, obtained by chemical modification of the natural metabolite A40926 produced by *Nonomuraea gerenzanensis* [18]. Vancomycin (Figure 1a) was the first GPA approved by the US Food and Drug Administration (FDA) in 1958, whereas teicoplanin (Figure 1b) was introduced to clinical use in Europe in 1988 and in Japan in 1998. Although discovered many decades ago, they both continue to be extensively used in clinical practice. Dalbavancin (Figure 1c) was approved in 2014 and designated as Qualified Infection Disease Product by the FDA, because of its potency, extended dosing interval, and unique dose regimen [15,19]. Although only two other GPAs are today in clinical practice (telavancin and oritavancin), dozens of newly discovered semi-synthetic vancomycin and teicoplanin analogues have been described in the last few decades, and many of them are potent and promising compounds against multidrug-resistant bacterial strains, as recently reviewed in [20,21]. The primary mechanism of action of GPAs is the binding to the D-Ala-D-Ala terminus of peptidoglycan precursors of bacterial cell wall, thus blocking mature cell wall assembly and, ultimately, leading to cell lysis [22,23]. In novel derivatives, the incorporation of a new membrane depolarization and disruption mechanism improves efficacy and evades resistance [24–27].

To our knowledge, this is the first report comparing the action of three clinically relevant GPAs when administered to *B. mori* larvae infected by *S. aureus*. Different infection parameters were monitored, including the survival rate of the treated larvae and multiple immunological markers, contributing to the

development of a robust and trustable infection model to be used along the discovery and development of novel GPAs.

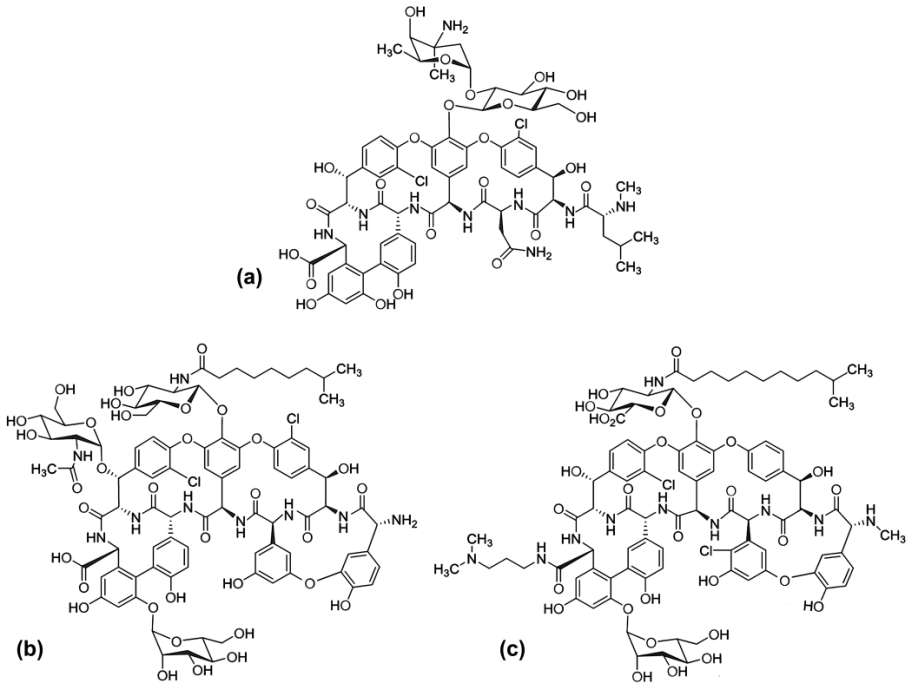

Figure 1. Structures of vancomycin (**a**), teicoplanin (**b**), and dalbavancin (**c**). For teicoplanin, the main component of the mixture used in clinical practice, i.e., component A2-2 bearing an 8-methylnonanoic (iso-C10:0) acid tail, is depicted.

2. Results

2.1. Rearing of B. mori Larvae at 37 °C

Under laboratory conditions, silkworms are usually reared at 25 °C [28]. With the aim to develop *B. mori* as an infection model comparable to mammalian ones, we first evaluated silkworm growth and development at 25 °C and 37 °C. We found that larval rearing at 37 °C did not produce any marked effect on the insects in the time window (from day 1 to day 4 of the fifth larval instar) considered in the experiments reported below. In fact, regardless of the incubation temperature, larvae remained mobile and actively feeding, they

progressively gained weight, and their survival was not affected (100% viability in both temperature conditions). Taking into account these results, the following experiments were performed at 37 °C.

2.2. Larval Survival after S. aureus Infection

Different cell concentrations of *S. aureus* ATCC 6538P (from 3 × 10 to 3 × 10^5 colony forming units (CFU) in 10 µL of injection volume) were used to infect silkworms, as described in Materials and Methods, and larval survival was monitored every 24 h for three days. In this time interval, control groups, i.e., uninjected larvae and larvae injected with only sterile physiological solution (0.6% w/v NaCl), showed normal feeding activity, and 100% of them remained viable after three days. As shown in Figure 2, 72 hours after the infection, the survival of larvae infected with 3 × 10 CFU and 3 × 10^2 CFU of *S. aureus* was 83% and 43%, respectively. Only 18% of the larvae infected with 3 × 10^3 CFU of *S. aureus* survived at 72 hours. Finally, all the larvae infected with 3 × 10^4 CFU and 3 × 10^5 CFU died in 48 hours. These data indicate an inoculum-dependent mortality, inferring that the lethal bacterial dose which killed 50% of the infected larvae (LD_{50}) corresponded to 3 × 10^2 CFU, as estimated by Probit analysis.

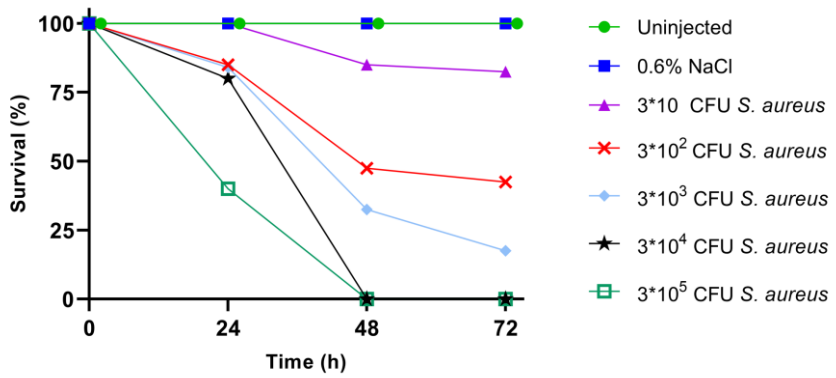

Figure 2. Survival rate of larvae infected with different concentrations of *S. aureus* (CFU, colony forming unit).

2.3. Cure of Infected Larvae by GPA Administration

Larvae infected with *S. aureus* at LD_{50} (3×10^2 CFU) were treated in parallel with the three GPAs selected for this study, i.e., vancomycin (Figure 3a), teicoplanin (Figure 3b), and dalbavancin (Figure 3c). The proper antibiotic dosage and its potential toxicity in the silkworm were initially set for vancomycin, taking into account the vancomycin antibiotic dosage (15–20 mg/kg body weight) recommended for treating bacterial infections as pneumonia, endocarditis, meningitis, etc., in humans [29]. To this purpose, three doses of vancomycin (8.75, 17.5, and 35 μg/g body weight) were injected in healthy larvae. At 72 h after the antibiotic treatment, the survival rate was 100% (data not shown), demonstrating that vancomycin was not toxic to *B. mori*. Consequently, the lowest dosage of vancomycin (8.75 μg/g body weight) was administered to healthy larvae and to infected larvae, in this last case two hours after *S. aureus* infection at LD_{50} (3×10^2 CFU). Results reported in Figure 3a indicate that vancomycin was not toxic to healthy larvae, and that antibiotic administration to infected larvae significantly improved their survival rate. In fact, 81% of the infected larvae survived in the first 24 hours, and this percentage decreased to 39% after 72 h, whereas the survival of larvae treated with vancomycin raised to 96% and 82% 24 h and 72 h after infection, respectively. Thus, we concluded that vancomycin can cure *S. aureus* infection in *B. mori* larvae when administered at the lowest tested dose.

The same evaluation of the potential toxicity of teicoplanin and dalbavancin in the silkworm was performed by injecting antibiotics in healthy larvae at the lowest active dose indicated for vancomycin (8.75 μg/g body weight). Similarly to vancomycin, the administration of these two GPAs did not affect larval survival, and 100% of the insects remained viable 72 h after the antibiotic

treatment (Figure 3b,c). In parallel, teicoplanin and dalbavancin were injected in infected larvae at the same concentration as vancomycin (8.75 μg/g body weight), two hours after *S. aureus* infection. The administration of teicoplanin increased the survival rate of infected larvae to 98% after 24 h and to 84% after 72 h from the injection (Figure 3b), whereas dalbavancin was even more effective considering that all the larvae survived 24 h after the antibiotic administration and 94% of them survived at 72 h (Figure 3c).

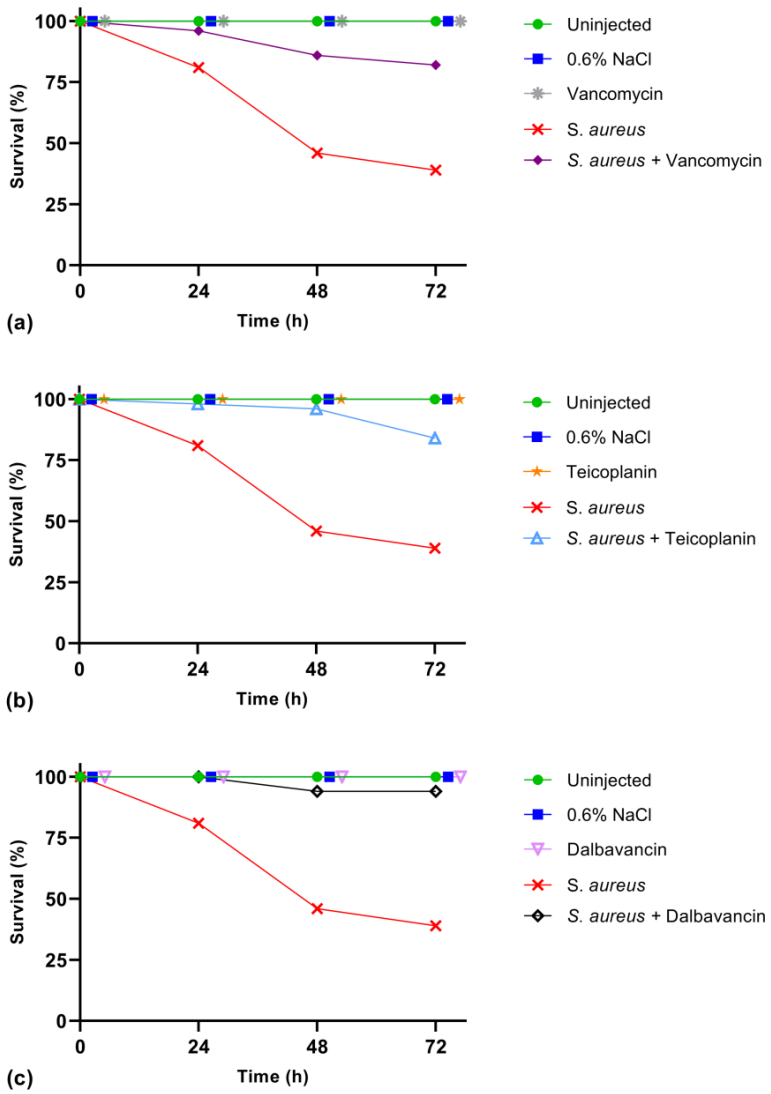

Figure 3. Survival rate of healthy larvae and larvae infected with *S. aureus* (3×10^2 colony forming units) when treated with vancomycin (**a**), or teicoplanin (**b**), or dalbavancin (**c**). The antibiotic dose was 8.75 μg/g body weight. Results reported in (**a**), (**b**), and (**c**) are from the same experiment run in parallel, using the same control groups (healthy larvae uninjected and injected only with saline solution) and infected larvae.

These results on the curing effect of the three GPAs in vivo are in agreement with their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) towards the *S. aureus* strain used in this study (Table 1). Dalbavancin (the novel semi-synthetic second-generation GPA) was more effective either in vivo or in vitro than teicoplanin and vancomycin, validating *B. mori* as a trustable infection model. These data are in fact consistent with those previously reported on these GPAs in preclinical and clinical studies in mice and humans, respectively [30,31] (see also Discussion).

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the three glycopeptide antibiotics used in this study towards *S. aureus* ATCC 6538P. Values represent the average of data from at least three independent experiments.

Antibiotic	MIC (μg/mL)	MBC (μg/mL)
Vancomycin	1	>128
Teicoplanin	1	128
Dalbavancin	0.5	16

2.4. GPA Effect on Bacterial Load in the Larvae

The effect of bacterial infection and GPA administration in *B. mori* was also followed by measuring the bacterial load in the silkworm body. As in previous

experiments, larvae were infected with *S. aureus* at LD_{50} (3×10^2 CFU) and the administration of each GPA at 8.75 μg/g body weight followed two hours after the infection. As shown in Figure 4, the bacterial load, measured as described in Materials and Methods, was comparable in the two control groups, i.e., uninjected larvae and larvae injected only with physiological solution, and this was likely due to the endogenous bacterial biota of the larvae. In the larvae infected with *S. aureus*, the bacterial load measured 24 h after the infection was very high (ca. 2.8×10^9 CFU/mL), indicating that *S. aureus* replicated quickly in the insect. The GPA administration (vancomycin in Figure 4) to the infected larvae reduced to zero the bacterial load in the insect body, confirming the antibiotic efficacy. Interestingly, the administration of the antibiotic alone to uninfected larvae exerted a bactericidal effect on the silkworm microbiota; thus, it would be interesting to further investigate the composition of endogenous microbiota and its response to GPAs and other antibiotics in the present *B. mori* infection model [32].

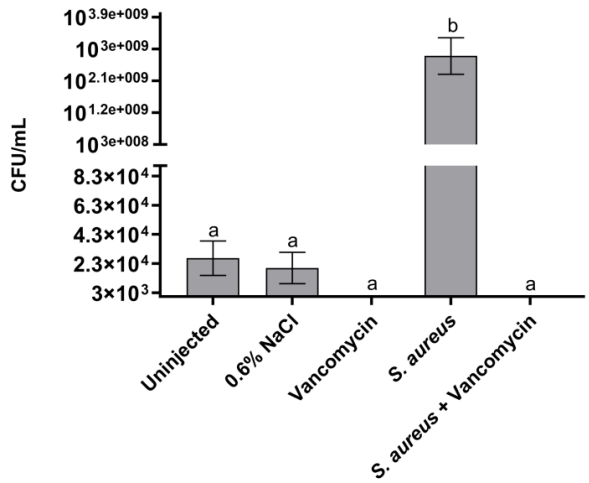

Figure 4. Analysis of bacterial load in control larvae (uninjected larvae and larvae injected only with physiological solution) and in healthy and infected larvae treated and untreated with vancomycin (8.75 μg/g body weight). Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

2.5. Immunological Markers of Infection

As other insects, *B. mori* possesses both humoral and cellular immune responses [33]. Defensive processes involving cell-mediated phagocytosis, encapsulation, and nodulation, are usually coupled with non-self-mediated melanization by activation of prophenoloxidase system (proPO), and antimicrobial peptide production triggered by bacterial infection [10]. In this study, we followed the cellular and humoral *B. mori* responses during *S. aureus* infection and GPA administration in the experimental model set up above (infection of the larvae with *S. aureus* at LD_{50}, followed 2 h later by GPA administration at 8.75 μg/g body weight).

2.5.1. Hemocyte Activity

A luminescence assay, based on ATP content quantification, was set up for evaluating the activity of hemocytes, which are responsible for the immune cell response against pathogen invasion [34]. Our results show a significant enhancement of ATP levels in infected silkworms (Figure 5), indicating an increased metabolic activity of immune cells induced by the exposure to *S. aureus*. The luminescence value in infected larvae treated with GPAs was comparable to that observed in uninfected control groups (i.e., uninjected larvae, larvae injected with the physiological solution, and larvae injected only with the GPA), suggesting that the antibiotic, killing *S. aureus*, blocks hemocyte activity/recruitment. The response in hemocyte activity following administration of vancomycin (Figure 5a), teicoplanin (Figure 5b), or dalbavancin (Figure 5c) was statistically comparable.

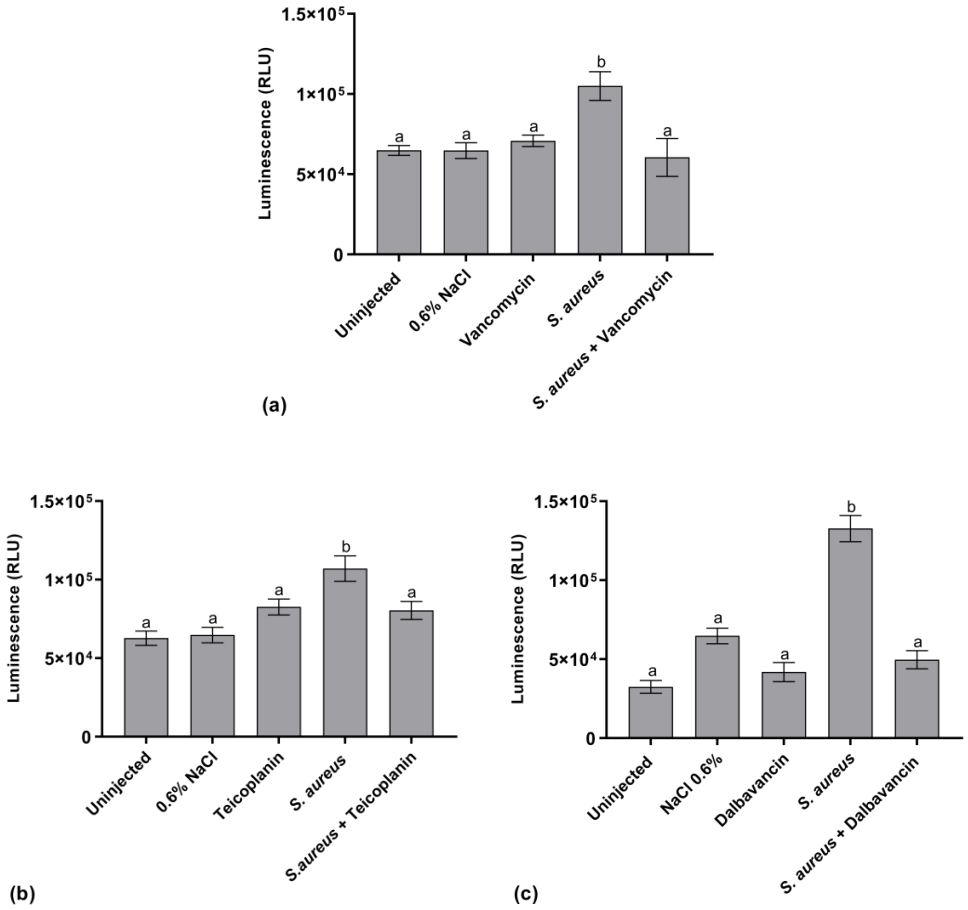

Figure 5. Luminescence indicating hemocyte activity in control larvae (uninjected larvae and larvae injected only with physiological solution) and in healthy and infected larvae treated and untreated with vancomycin (**a**), teicoplanin (**b**), and dalbavancin (**c**) at 8.75 μg/g body weight. Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

2.5.2. AMP Expression

Antimicrobial peptides (AMPs) are naturally occurring molecules produced as a first line of defense against pathogenic infections. They play an essential role in those organisms that base their defense only on innate immune response, such as insects [35]. In the silkworm, mRNA synthesis of several AMPs, as cecropins, is highly upregulated as soon as the larvae undergo bacterial infection [36].

Herein, we monitored mRNA transcription of *BmCECE* and *BmCECB1* genes coding for silkworm cecropins, which are reported to be active against Gram-positive and Gram-negative bacteria [35]. For both genes, no significant differences in mRNA levels were observed in all the control groups of larvae. Apparently, the expression level of the two AMPs increased in the infected larvae, as expected, and then it was (*BmCECB1*), or it was not (*BmCECE*), reverted to the basal level by GPA administration (vancomycin in Figure 6), but these results were not statistically significant. Further investigations are needed to understand the relatively low and variable level of AMP gene expression observed in these experiments after infection (see Discussion), and how antibiotic administration might counteract it.

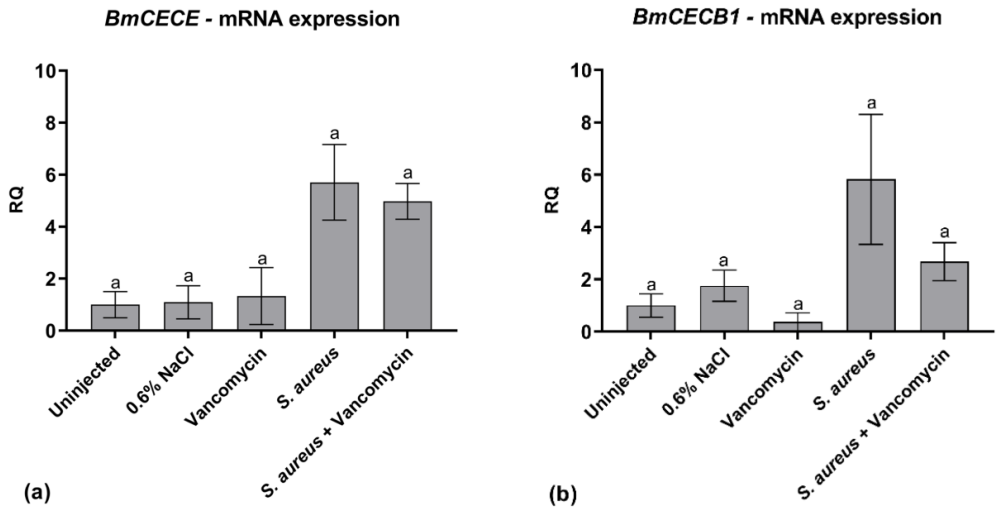

Figure 6. Quantitative reverse transcription PCR (qRT-PCR) analysis of *BmCECE* (**a**) and *BmCECB1* (**b**). mRNA levels in control larvae (uninjected larvae and larvae injected only with physiological solution) and in healthy and infected larvae treated and untreated with vancomycin (8.75 μg/g body weight). Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

2.5.3. Activation of the proPO System

In insects, proPO system activation and the consequent synthesis of melanin are considered important mechanisms of the immune response. Melanization is often induced by pathogens that enter the host [37]. Herein, the activation of the proPO system was monitored by measuring the phenoloxidase (PO) enzyme activity, using the enzymatic assay described in Materials and Methods. As shown in Figure 7, no significant variations in the PO relative activity were measurable in the hemolymph of the control groups. Conversely, a marked reduction of PO activity occurred following the infection of larvae with *S. aureus* at LD_{50}, likely indicating a drastic impairment of immune response in the presence of high bacterial load in the hemocoel. The administration of vancomycin (Figure 7a) or teicoplanin (Figure 7b) to infected larvae restored the basal level of PO activity. Indeed, after dalbavancin administration, the level of PO activity was enhanced if compared to control conditions (Figure 7c). Thus, restoration of PO activity seems dependent on the curing effect of antibiotics. Although the physiological meaning of these observations remains to be further investigated, the PO assay might be promising for evaluating the efficacy of different GPAs.

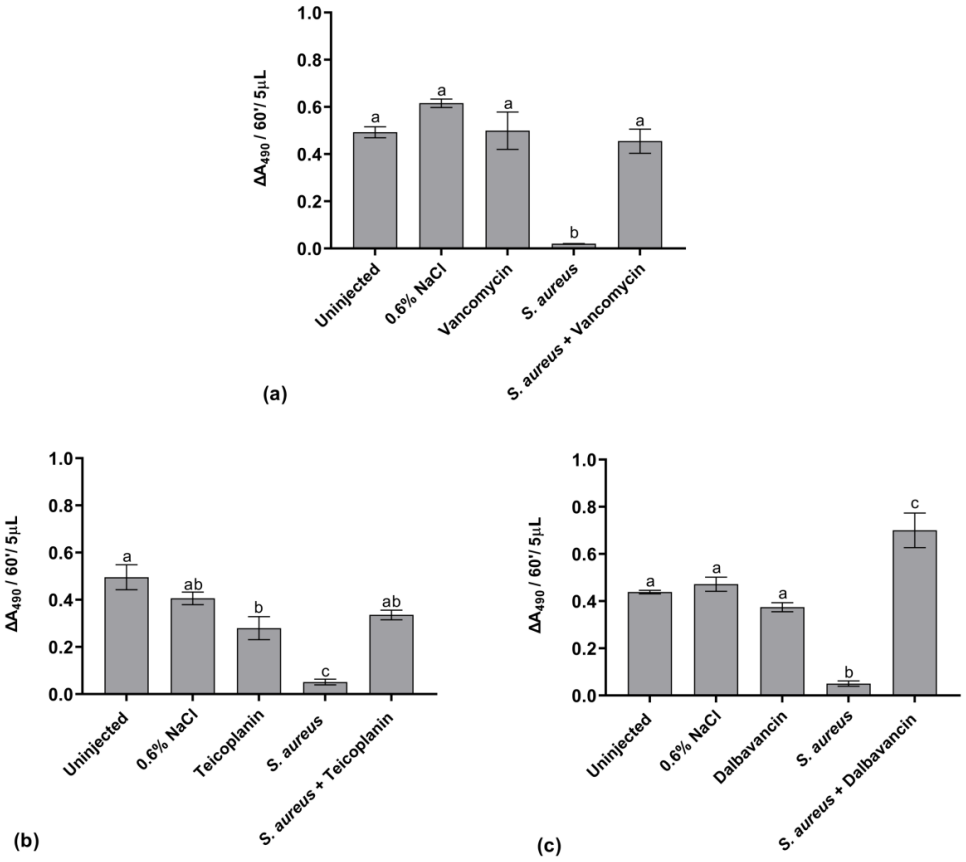

Figure 7. Analysis of phenoloxidase (PO) activity in control larvae (uninjected larvae and larvae injected only with physiological solution) and in healthy and infected larvae treated and untreated with vancomycin (**a**), teicoplanin (**b**), and dalbavancin (**c**) at 8.75 μg/g body weight. Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

3. Discussion

B. mori has long been used for silk production [38] and it represents an established biological model for studying insect physiology and immunity [39,40]. More recently, *B. mori* has been proposed as a model of infection alternative to mammalian ones [41,42]. Larvae infected by those pathogenic bacteria or fungi that are fatal in humans generally die, but their infection could

be counteracted by antibiotic administration [12,43]. Efficacy of antibacterial agents belonging to different chemical classes, including kanamycin, tetracycline, fluconazole, etc., was previously tested in silkworm larvae [41,42,44,45]. Most of these studies monitored the larval survival, the proliferation of the infecting bacterium in the larval body, and the pharmacokinetics of antimicrobial agents [41,42,46]. Effects on the innate immunity of silkworm remained largely unexplored. In this work, we studied *B. mori* as an infection model to evaluate the efficacy of old and novel GPAs, monitoring a set of markers spanning from larval survival to cellular and humoral immunological responses.

First, we demonstrated that *B. mori*—as in the case of *G. mellonella*, which is already used as an invertebrate infection model [8,47]—has larval stages that can survive at 37 °C, allowing the study of microbial virulence under human basal temperature. Although a negative effect of high temperatures on silkworm survival rate was previously documented [48], herein, we set up tightly controlled experimental conditions for larval growth, such as the exposure to 37 °C for a short timeframe of the fifth larval instar, and the use of a germ-free artificial diet to feed the larvae [28].

Silkworm reared on artificial diet, instead of mulberry leaves, show a reduction of the gut microbiota diversity [49]. Larvae with such microbiota are less prone to events of gut flora imbalance and secondary bacterial septicemia, which could be induced by high temperatures, and that can decrease silkworm resistance, immunity, and survival [50]. Additionally, this simplified endogenous bacterial biota might favor a better evaluation of bacterial virulence in the silkworm, thus improving the robustness of our infection model.

Our results of larval response to the infection showed a direct correlation between *S. aureus* inoculum and the survival rate. Overall, we observed a lower

survival rate of infected larvae if compared to previous studies [41,42], in which silkworms were reared at 25 °C. The different temperature at which larvae where reared could explain this dissimilarity. Indeed, the rearing temperature used in this study (i.e., 37 °C) corresponds to the optimal growth temperature for *S. aureus*, determining a higher bacterial proliferation in the hemocoel and compromising larval vitality just a few hours after the infection. The administration of GPAs at dosages comparable to the ones used in humans was effective in treating infected larvae, and in significantly reducing the bacterial load in the larval body. The silkworm infection model, as set in our experimental design, turned out to be predictive of the higher and more prolonged potency of the second generation dalbavancin compared to the first-generation vancomycin and teicoplanin. Although future investigations will focus on varying GPA dosage and/or administering them in repeated injections, the in vivo and in vitro data herein reported are in agreement with those previously highlighted during preclinical studies in rats and mice, and clinical trials in humans [19,31]. When used for treating staphylococcal endocarditis in rats and septicemia in immunocompetent and neutropenic mice, a single daily dose of dalbavancin was found to be equal, or even more effective, than multiple doses of either teicoplanin or vancomycin [30]. A higher efficacy of dalbavancin was also demonstrated during phase II and phase III clinical trials in adult patients with catheter-related staphylococcal bloodstream infection, or affected by skin and soft-tissue infections [51,31].

In the second part of our work, we investigated the activation of innate cellular and humoral responses of *B. mori* to *S. aureus* infection. Our data confirmed the primary role of hemocytes against pathogen invasion [34] and indicated that the activity of these cells might be used as a marker for monitoring bacterial infection and GPA administration in silkworm. The involvement of the humoral response

was assessed studying variations in PO activity and expression of AMPs [52,53]. The relative activity of PO turned out to be a good indicator for monitoring infection progress and for evaluating the curing activities of diverse GPAs. Indeed, the analyses of gene expression pattern for silkworm cecropins did not provide the expected results. In fact, although an apparent increase in the transcriptional rate was observed in infected silkworms compared to controls, the difference registered among the experimental groups could not be considered statistically significant. Romoli et al. (2017) demonstrated that AMP production after bacterial infection depends on the silkworm strain and pathogen in use [36]. AMP production is lower in Chinese and Japanese silkworm strains. The reduced expression level of the two AMP genes that we observed 24 h after infection could be due to the use of polyhybrid silkworms, derived from a four-way crossbreed between a Chinese and a Japanese strain. However, we cannot exclude that the unexpected variations of AMP mRNA expression levels are somehow due to the high rearing temperature, as previously shown for other genes [54].

Although further investigations on the role and interaction of the different components concurring to the innate immunity of *B. mori* are needed, our work adds novel evidence on the current knowledge. We could identify useful tools for monitoring bacterial infection and evaluating GPA therapeutic potential in this simple, inexpensive, and easy-to-handle insect model. Measurements of larval survival rate, hemocyte activity, and PO enzyme activity in infected larvae treated and untreated with different antibiotics were easy to perform and relatively cheap; moreover, they required low amount of GPAs to be tested, and allowed us to assess the potency of three clinically important GPAs. Thus, this study contributes to validating *B. mori* as an alternative animal infection model for screening old and novel GPAs, potentially reducing the number of mammals

to be used in the preclinical phases of drug discovery and development. This outcome may be relevant, considering that novel GPAs are urgently needed to overcome emerging antimicrobial resistance and prolong the clinical longevity of this important antibiotic class [14,15,17,20,21].

4. Materials and Methods

4.1. Experimental Model

Larvae of *B. mori* [polyhybrid (126 × 57) (70 × 90)], provided by CREA-AA, Sericulture lab (Padova, Italy), were reared in groups of ten in glass Petri dishes (180 mm × 30 mm) at 25 ± 0.5 °C under a 12 : 12 h light : dark period and 70% relative humidity. Larvae were fed on artificial diet [28] until the end of the IV larval instar. After animals had ecdysed to the V larval instar, silkworms were synchronized [55] and fed with a daily amount of antibiotic-free and germ-free artificial diet, as previously reported in [56]. All the experiments were performed with larvae at fifth instar.

4.2. B. mori Rearing at 37 °C

After the last larval molt, silkworms were incubated at 37 ± 0.5 °C and grown as described in section 4.1. Insect growth, mortality, and larval behavior were assessed daily during the fifth larval instar and data were compared to those obtained from larvae grown at 25 ± 0.5 °C. Thirty larvae were used for each temperature condition.

4.3. Bacterial Strains and Culture Conditions

For strain reactivation, *S. aureus* subsp. Rosenbach ATCC 6538P was grown overnight at 37 °C under shaking at 200 rpm (revolutions per minute) in 10 mL of MHB2 (Müller Hinton Broth 2, VWR International S.r.l., USA). 1 mL of culture

was centrifuged for 10 minutes at 1900× g and 4 °C, supernatant was discharged, and cell pellet resuspended in sterile physiological solution (0.6 % w/v NaCl) to reach a concentration of 3 × 10^8 CFU/mL. The volume of physiological solution to be added was calculated by measuring the optical density of the culture at 600 nm (OD600nm) and taking into consideration that one unit of OD600nm corresponds to ca. 2.4 × 10^8 CFU of *S. aureus* per mL. Serial dilutions with sterile physiological solution were then made to obtain the bacterial suspensions with the desired cell concentration.

4.4. Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)

MICs of vancomycin (Sigma-Aldrich, USA), teicoplanin (Sigma-Aldrich, USA), and dalbavancin (kindly gifted by Sanofi, Italy) toward *S. aureus* were determined by the broth dilution method, according to the guidelines of the Clinical and Laboratory Standards Institute [57]. The antibiotics were prepared by dissolving the corresponding powder in deionized water, filtered with a cut-off of 0.22 μm, and finally brought to appropriate dilution with MHB2. Ca. 5.0 × 10^5 bacterial cells in exponential growth were inoculated in MHB2, together with increasing concentrations of the antibiotics. MICs were defined as the minimal antibiotic concentration at which no turbidity could be detected after incubation for 20 h at 37 °C and 200 rpm. For calculating MBCs, 0.1 mL of bacterial cultures used for the MIC test were plated on MHA (Müller Hinton Agar, VWR International S.r.l., USA), then incubated at 37 °C for 24 h. MBCs were the lowest antibiotic concentrations, at which no growth could be observed.

4.5. Injection of Larvae and Collection of Hemolymph

For all the experiments described below, larvae at the second day of the fifth instar were injected in the second right proleg by using autoclaved Hamilton 1702 LT 25 μL syringes (Hamilton, USA). Injections were performed under sterile hood. Hemolymph was collected from the larva by cutting the second left proleg.

4.6. Determination of Lethal Dose 50 (LD_{50}) for S. aureus

To determine the LD_{50}, silkworms were injected with 10 μL containing different concentrations of *S. aureus* cell suspension (3×10^3, 3×10^4, 3×10^5, 3×10^6, and 3×10^7 CFU/mL), and their mortality was monitored every 24 h for three days. Uninjected larvae and larvae injected with 10 μL of physiological solution (0.6% w/v NaCl) were used as controls. Forty larvae for each experimental condition were used. Larvae were considered dead when there was no reaction after stimulation with a plastic tip. LD_{50} was defined as the concentration of bacteria at which 50% of animals died within 72 h after the infection. LD_{50} and fiducial limits were calculated by Probit analysis [58].

4.7. GPA Administration

To evaluate the toxicity of vancomycin on the survival of healthy larvae, 10 μL of antibiotic at 8.75, or 17.5, or 35 μg/g body weight diluted in sterile physiological solution were injected and larval mortality was monitored for 72 h. Uninjected larvae and larvae injected with 10 μL of physiological solution (0.6% w/v NaCl) were used as controls. Thirty larvae were used for each experimental group. To evaluate the effects of antibiotics on the survival of infected silkworms, larvae were injected with 10 μL of *S. aureus* at LD_{50}, followed by injection 2 h later of 10 μL of vancomycin, or teicoplanin, or dalbavancin at 8.75 μg/g body weight. In preliminary trials, a second injection

of 10 μL of physiological solution in infected larvae was tested in parallel to antibiotic injection, and it did not alter their physiological state. Consequently, untreated (uninjected) larvae, larvae injected once with physiological solution (10 μL of 0.6% w/v NaCl), and healthy larvae injected with 10 μL of antibiotic were used as controls groups. Larval mortality was monitored for 72 h. Fifty larvae were used for each experimental condition.

To evaluate the bacterial load and the immunological markers, experimental groups were as follows: untreated larvae, larvae injected with 10 μL of 0.6% w/v NaCl, larvae injected with 10 μL of antibiotic (i.e., vancomycin, teicoplanin, or dalbavancin) at 8.75 μg/g body weight, larvae injected with 10 μL of 3×10^5 CFU/mL of *S. aureus*, larvae injected with 10 μL of 3×10^5 CFU/mL of *S. aureus* and, two hours later, with 10 μL of antibiotic at 8.75 μg/g body weight). After injections, silkworms were reared at 37 °C. 24 h after the first injection, surviving larvae were analyzed, as described below (Sections 4.7.1–4.7.4).

4.7.1. Hemocyte Activity

Hemolymph was collected from the larvae and diluted 1:50 with Saline Solution for Lepidoptera (sucrose 210 mM, KCl 45 mM, Tris-HCl 10 mM, pH 7.0). The viability of hemocytes was evaluated by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, USA). Briefly, 100 μL of diluted hemolymph and 100 μL of CellTiter-Glo reagent were mixed into a 96-well plate, and then incubated for 5 min at room temperature on an orbital shaker. Luminescence was measured using an Infinite F200 96-well plate-reader (Tecan, Switzerland). Ten surviving larvae for each experimental group were analyzed.

4.7.2. AMP Expression

The fat body was isolated from larvae, cleaned from tracheae, and immediately frozen in liquid nitrogen. RNA was extracted from 20–30 mg of tissue with Trizol Reagent (Life Technologies, USA). Genomic DNA contamination was removed using TURBO DNA-free Kit (Life Technologies, USA) and the RNA quality was verified through gel electrophoresis. Primers used for quantitative reverse transcription PCR (qRT-PCR) are indicated in Table 2. BmRP49 was used as housekeeping gene, to calculate the relative expression of the cecropin genes [59]. iTaq Universal SYBR Green Supermix (Bio-Rad, USA) and a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) were used to perform PCR. Relative expression of the genes was calculated with the $2^{-\Delta\Delta Ct}$ method. The efficiency of the amplification reaction of each gene was adjusted to be in the range of 90%–105%. Fifteen surviving larvae for each experimental group were analyzed.

Table 2. Primer sequences used in this study.

Gene	Accession number	Primer sequences
BmRP49	NM_001098282.1	F: AGGCATCAATCGGATCGCTATG R: TTGTGAACTAGGACCTTACGGAATC
BmCECE	DQ233467.1	F: GTGTGTGCGAGCGTTATGGC R: CCCATGAGCGATGGTCGCC
BmCECB1	BGIBMGA000024-RA	F: TTCGCTCTGGTGCTGGCTTTG R: GGCCCGCTTTGACGATGCC

4.7.3. Activation of Prophenoloxidase System

The activation of the prophenoloxidase system was evaluated by monitoring the activity of the enzyme phenoloxidase. Hemolymph was collected from larvae and 5 μL were added to a 1 mL solution containing 8 mM L-Dopa (L-3-4

dihydroxyphenylalanine) (Sigma-Aldrich, USA) in Tris-HCl 10 mM. The relative activity of phenoloxidase (formation of dopachrome) was evaluated as an increase in optical density over time, at 490 nm for 60 min. The activity was registered using a V-560 double-beam spectrophotometer (Jasco, USA). Nine surviving larvae for each experimental group were analyzed.

4.7.4. Bacterial Load

Larvae were surface sterilized with 70% v/v ethanol. They were then placed in disposable 15 mL centrifuge tubes and homogenized using a Potter-Elvehjem PTFE pestle according to [60]. 100 μL of serial dilutions of the homogenate (from 10−1 to 10−5, in sterile physiological solution) were plated onto MHA plates and incubated at 37 °C. The number of colonies (as CFU/mL) was calculated after 24 h. All the procedures were performed under sterile hood. Five surviving larvae for each experimental group were analyzed.

4.8. Statistical Analysis

Statistical analysis was performed using ANOVA, followed by Tukey's Honestly Significant Difference (HSD) test (significance $p < 0.05$).

5. Conclusions

Our results show the usefulness of in vivo-mimic infection of *B. mori* larvae by *S. aureus* to assess the therapeutic potential of old and novel GPAs. Immunological markers tested herein demonstrated to be promising for evaluating the efficacy of different GPAs. In particular, the novel second generation dalbavancin was confirmed to be more effective than the first-generation drugs vancomycin and teicoplanin in curing *S. aureus* infection. Our hope is that this insect infection model might accelerate the discovery and development of novel GPAs, which are urgently needed to prolong the clinical longevity of such important class of

life-saving drugs. This model could be introduced in preclinical phases prior to the use of mice and might help in overcoming bottleneck steps between the in vitro and in vivo experimental phases.

Author Contributions: Conceptualization, F.M. and G.T.; Data curation, A.M. and A.S.; Investigation, A.M., F.B., M.M. and A.S.; Methodology, F.B., M.F.B. and M.M.; Supervision, F.M. and G.T.; Validation, A.M. and F.B.; Writing – original draft, A.M., F.B., M.F.B. and G.T.; Writing – review & editing, F.B., M.F.B., S.C., F.M. and G.T. All authors have read and agreed to the published version of the manuscript.

Funding: The work was partially supported by FAR 2018–2019 (Fondi di Ateneo per la Ricerca, University of Insubria) to G.T. and F.M.

Acknowledgments: The authors are grateful to Silvia Caccia for assistance with Probit analysis. We acknowledge the support of CIB (Consorzio Interuniversitario per le Biotecnologie) to F.B. A.M. is a Ph.D. student of the 'Life Science and Biotechnology' course at University of Insubria.

Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 2

An in-depth characterization of *Hermetia illucens* immune system

Daniele Bruno [1,+], Aurora Montali [1,+], Maristella Mastore [2], Maurizio Francesco Brivio [2], Annalisa Grimaldi [1], and Gianluca Tettamanti [1,3,*]

[1] Department of Biotechnology and Life Sciences, University of Insubria, 21100 Varese, Italy; d.bruno1@uninsubria.it (D.B.); a.montali@uninsubria.it (A.M.); annalisa.grimaldi@uninsubria.it (A.G.); gianluca.tettamanti@uninsubria.it (G.T.).
[2] Department of Theoretical and Applied Sciences, University of Insubria, 21100 Varese, Italy; maristella.mastore@uninsubria.it (M.M.); maurizio.brivio@uninsubria.it (M.F.B.).
[3] Interuniversity Center for Studies on Bioinspired Agro-environmental Technology (BAT Center), University of Napoli Federico II, 80055 Portici, Italy
* Correspondence: gianluca.tettamanti@uninsubria.it; Tel.: +39 0332-421312
+ These authors contributed equally to the work

Keywords: immune system, black soldier fly, hemocytes, phenoloxidase system, lysozyme, antimicrobial peptides

In preparation

Abstract: The continuous increase in food consumption and the consequent production of an increasing amount of organic waste are leading to the establishment of circular economy supply chains based on the use of insects for waste valorization. In this setting the larvae of black soldier fly (BSF), *Hermetia illucens* (Diptera: Stratiomyidae) are increasingly drawing attention. In fact, this saprophagous insect can be reared on a wide range of substrates and, thanks to their high nutritional value, BSF larvae are suitable for the production of feedstuff. However, the environment in which BSF larvae grow can bring them in contact with a high range of microorganisms. For this reason they should have evolved a sophisticated immune response, but to date the information about this aspect are still fragmentary.

In the present work, we characterized the cellular and humoral response of *H. illucens* larvae infected with a mix of Gram-positive and Gram-negative bacteria. In particular, we performed a morphological analysis of hemocytes, evaluated phagocytosis, and investigated the encapsulation processes. Moreover, we analyzed key components of the humoral immune response (i.e., phenoloxidase system, lysozyme, and antimicrobial peptides). We observed that phagocytosis, and encapsulation rapidly occur after the injection of the larvae with the bacterial mix. Conversely, the humoral response is activated later and mainly involves lysozyme activity and AMP production, while proPO system is inhibited when the larva is exposed to low bacterial concentrations. The results obtained in this study allowed to obtain an in-depth characterization of the immune system of *H. illucens* that could be used as a starting point for future studies that aim at modulating the immune response through external factor, such as the diet, to enhance the resistance to pathogens and the health status of BSF larvae in mass rearing activities.

1. Introduction

Nowadays, the production of feed for farmed animals is becoming an increasingly demanding challenge. In fact the feed industry takes up land and uses large quantities of water and energy, thus impacting heavily on the environment and, at the same time, subtracting resources that could be used for human nutrition (Wang and Shelomi, 2017). Furthermore, due to the continuous increase in the world population, the protein requirement for livestock will significantly increase. Therefore, it is essential to identify alternative sources of proteins to support this request (Sachs et al., 2010). At the same time, it is also necessary to consider that every year one third of the food that is globally produced is wasted (Gustavsson et al., 2011). In recent years the possibility of rearing saprophagous insects on organic matter for the production of proteins that can be used for feed formulation is attracting increasing interest. The exploitation of insect larvae for the bioconversion of organic waste would not only reduce waste, but could also provide a valid alternative for feed production, thus generating a circular economy supply chain (Cappellozza et al., 2019). In this scenario, the larvae of *H. illucens* (Diptera: Stratiomyidae) might play a key role. Native to the Americas (Wang and Shelomi, 2017) but now worldwide distributed, the black soldier fly (BSF), *H. illucens*, is a saprophage insect whose larvae can grow on a wide variety of organic matters (Čičková et al., 2015; Wang et al., 2017). It is well known that these larvae are able to reduce low-quality substrates, as vegetable and fruit waste, animal manure, and urban organic waste, and their proteins and lipids can be used for the formulation of feedstuff for livestock, poultry, and aquaculture (Kalová et al., 2013; Čičková et al., 2015; Rehman et al., 2019; Kierończyk et al., 2020; Proc et al., 2020). Moreover they can also be exploited for the production of biodiesel and bioplastics (Wang et al, 2017; Barbi et al; 2019), as well as for the isolation of chitin and chitosan (Liu et al, 2019),

enzymes able to degrade chitin and lignin (Müller et al., 2017), and antimicrobial peptides (Buchon et al., 2014; Vogel et al, 2018).

The rearing procedures for BSF (e.g., composition of the substrate, temperature, humidity) can significantly impact on the quality of the final product (Wang and Shelomi, 2017). However, BSF-based applications, most of which involve the use of decaying waste substrates, bring this insect into contact with different potential pathogens that can challenge insect health and performance. Accordingly, the European legislation sets limits on the waste type that can be used to feed BSF larvae to avoid possible contaminations of the meal obtained from this insect, thus preventing the possible introduction of pathogens in the food production chain (Nguyen et al., 2015; European Commission, 2017; Zdybicka-Barabas et al., 2017).

The immune system of insects relies on a sophisticated set of cellular and humoral innate mechanisms that are rapidly activated in the presence of infections. The main actors of the cellular response are the hemocytes (Lavine and Strand, 2002). In Diptera, and in particular in *Drosophila,* hemocytes were classified in prohemocytes, plasmatocytes, crystal cells, and lamellocytes (Lanot et al., 2001; Wertheim et al., 2005). They are involved in phagocytosis, encapsulation, and nodulation, through which they can recognize, bind, and destroy *non-self* particles (Lavine and Strand, 2002). On the other hand, the humoral response is promptly triggered after the interaction between Pattern Recognition Receptors (PRRs), that are located on the surface of insects cells, and Pathogen-associated Molecular Patterns (PAMPs) as peptidoglycan, lipopolysaccharide, and β-1,3 glucans that are expressed by the pathogens. The humoral response includes the phenoloxidase system (PO), an enzymatic cascade whose activation culminates with hemolymph clotting and melanin production, antimicrobial peptides, lysozyme, and Reactive Oxygen Species

(Tsakas and Marmaras., 2010; Rosales, 2017). In particular, the phenoloxidase system plays a crucial role in insect immunity. The activation of this enzyme allows to contain the spread of pathogens in the hemolymph thanks to the production of melanin and cytotoxic intermediates that are deposited around the *non-self* agent (Nappi and Christensen, 2005). Besides the proPO system, the binding of PAMPs to PRRs leads to the synthesis of antimicrobial peptides (AMPs). These inducible proteins are mainly produced by the fat body and, depending on their function, they can act against Gram-positive or Gram-negative bacteria, or both (Lemaitre e Hoffmann, 2007). Finally, constitutively expressed lysozymes, thanks to their muramidase activity, are able to cleave the peptidoglycan of Gram-positive bacteria (Lemaitre e Hoffmann, 2007).

To date, very few information is available on the immune response of *H. illucens*. Most of the papers focused the attention on the isolation and characterization of AMPs (Elhag et al., 2017; Vogel et al., 2018), and only a preliminary study on the immune system of this insect was done (Zdybicka-Barabas et al., 2017), in which they described some components of the humoral response, completely overlooking the cellular ones.

In this paper we analyzed and compared the cellular and humoral response of *H. illucens* subjected to a bacterial infection by administering Gram-negative and Gram-positive bacteria (*E.coli* and *M.luteus*, respectively) to the larvae. In particular, we characterized the different types of hemocytes, investigated their activity in phagocytosis and encapsulation, evaluated the antimicrobial activity of the humoral and cellular response, analyzed the activity of the proPO system and lysozyme, and quantified the expression of AMPs.

Besides a characterization of the main hemocyte types, our results demonstrate that the two components of the immune system have different timing of

activation: in particular, phagocytosis and encapsulation are activated promptly after the infection, while the humoral components act more slowly.

To the best of our knowledge, this study represents the first in-depth characterization of the immune response of *H. illucens*. The knowledge on the BSF defense mechanisms and the modulation of the immune response by environmental factors, such as the diet or the temperature, could allow the optimization of the resistance of the larvae to pathogens and of their health during mass rearing, in the next future.

2. Materials and methods

2.1. Insect rearing

Larvae of *H. illucens* used in this study were obtained from a colony established in 2015 at the University of Insubria (Varese, Italy) starting from larvae purchased from a local dealer (Redbug, Milan, Italy).

After the deposition, eggs were collected in Petri dishes (9 cm x 1.5 cm) and maintained at 27 ± 0.5 °C until hatching, according to the procedures described in Pimentel et al. (2017). After the weaning phase, 300 larvae were transferred to a plastic container (16 x 16 x 9 cm) and fed with a standard diet for Diptera (Hogsette, 1992) (50% wheat bran, 30% corn meal, and 20% alfalfa meal mixed at a ratio of 1:1 dry matter:water), which was renewed every two days. Insects were reared in the dark at 27 ± 0.5 °C and 70 ± 0.5 % humidity. Once the pupal phase was reached, insects were separated from the substrate and transferred in a cage (70 x 70 x 120 cm) until adult eclosion. Flies were kept at 30 ± 0.5 ° C, 70 ± 5 % relative humidity and with a photoperiod of 12:12 hours of light: dark photoperiod, obtained by using an Osram 36W/765 fluorescent neon lamp (Osram, Regensburg, Germany).

2.2. Bacterial strains

Larvae were infected with *E. coli* (Strain K12, Sigma-Aldrich, USA) and *M. luteus ATCC No. 4698* (Sigma-Aldrich). For strain reactivation, bacteria were grown in 10 ml of LB (Luria-Bertani) (Sigma-Aldrich) overnight at 37 °C under shaking at 160 rpm. 1 ml of both cultures was centrifuged at 1620 x *g* for 15 minutes and the supernatant was discharged. Cell pellets were washed three times with phosphate buffer (38 mM KH_2PO_4, 61.4 mM K_2HPO_4, pH 7.4), centrifuging at every step at 1620 x *g* for 15 minutes at 4 °C. Finally, cells were resuspended in Phosphate Buffered Saline (PBS, 138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4) to reach a concentration of $2x10^5$ CFU/ml. The optical density of the culture at 600 nm (OD600nm) was measured to calculate the volume of PBS to be added, considering that one unit of OD_{600nm} corresponds to 4.12×10^8 CFU/ml of *E. coli* and 1.83×10^7 CFU/ml of *M. luteus*.

2.3. Injection of larvae and collection of hemolymph

For all the experiments described below, *H. illucens* larvae at the last larval instar were injected with 5 µl of a mix (1:1, v/v) of both bacteria at a final concentration of 10^5 CFU/ml by using Hamilton 700 10 µl syringe (Hamilton, USA). The injection of a mixture of Gram-positive and Gram-negative bacteria into the larvae was made in order to obtain a simultaneous response of the immune system against both strains.

Larvae were washed with tap water to remove the excess of diet and subsequently sterilized with 0.5% sodium hypochlorite (in tap water, v/v) and 70% ethanol (in distillated water, v/v).

Injections were performed between the third last and penultimate metamere. After the injection, the larvae were maintained in sterile Petri dishes (14 cm x 1.5 cm) at 27 ± 0.5 °C and 70 ± 0.5% humidity without feeding substrate to avoid a

possible infection due to the bacteria present in the diet. As a comparison, for most of the analyses (i.e., total hemocyte count, lysozyme activity, and RNA expression), infected larvae were reared with standard diet in plastic containers. Taking into consideration that the aim of the work was to analyze the immune response triggered by a possible lesion of the insect body, uninjected larvae (naïve) were considered as control group. To exclude potential effects of the PBS used to prepare the bacterial mix on the health of the insects and on the induction of some immune markers (i.e., cell count, gene expression and lysozyme activity), larvae were punctured with a sterile needle or injected with 5 µl of sterile PBS and the hemolymph was analyzed 14 hours after the injection.
Hemolymph was collected by piercing larvae, previously anesthetized on ice, with a sterile needle.

2.4. Determination of the bacterial concentration

To determine the bacterial amount to be used for the infections, larvae were injected with 5 µl of different concentrations of *E. coli*/*M. luteus* mix (10^4, 10^5, 10^6, 10^7, 10^8, 10^9 CFU/ml) and their mortality was monitored every 24 hours for three days. Twenty-five larvae for each condition were used and the analysis was performed in triplicate.

2.5. Analysis of bacterial load

To determine the bacterial load (as CFU/ml) present in *H. illucens*, the hemolymph of control and immunized larvae was collected 6, 14, 24, and 48 hours after the infection. Samples were diluted 1:100 with sterile PBS and then plated onto 20 mL of LB broth agar (Sigma-Aldrich). The plates were incubated at 37 °C for 24 hours and subsequently the number of colonies was counted. The CFU/ml was calculated as $CFU/ml = n° colonies \times dilution\ factor$.

2.6. AMP activity: CFU count by track-dilution method

Larvae were injected and maintained for 6, 14, 24, and 48 hours under starvation as described in paragraph "Injection of larvae and collection of hemolymph". After the collection of hemolymph, specimens were centrifuged at 250 x *g* for 5 minutes to remove the hemocytes. 10 µl of samples were incubated for 3 hours with 90 µl of 10^6 CFU/ml *E. coli* or *M. luteus*. A mix of 10^6 CFU/ml *E. coli* or *M. luteus* and sterile PBS was used as control. Samples were serially diluted and then 10 µl of samples were dropped onto an agar plate. Plates were tilted to gravitate downward the droplets and then incubated overnight at 37 °C. The number of colonies (CFU) in each plate was counted and the effective dilution was calculated as $CFU/ml = n° colonies \ x \ 10 \ x \ dilution \ factor$.

2.7. Analyses of the cellular response

2.7.1. Total hemocyte count

To quantify the hemocytes, hemolymph samples were extracted 6, 14, 24, and 48 hours after the bacterial infection. Total hemocyte counts (THCs) were performed by loading diluted hemolymph (10 µl hemolymph added to 90 µl 0.4% Trypan blue, ThermoFisher, USA) into FAST READ 102 counting chambers (Biosigma S.R.L., Italia). Groups of five larvae from three different batches were used for each condition.

2.7.2. Transmission electron microscopy

Hemolymph was collected by cutting the apical part of naïve larvae, and then fixed in 1% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4 (1:1 v/v) overnight at 4 °C. Samples were centrifuged at 200 x *g* for 10 minutes and then postfixed in 2% osmium tetroxide in 0.1 M Na-cacodylate buffer for 20 minutes. After a standard dehydration in ethanol series, samples were embedded in an

Epon–Araldite 812 mixture. Thin sections (70 nm) were obtained with a Leica Reichert Ultracut S (Leica, Germany) and stained with lead citrate and uranyl citrate. Finally specimens were observed with a JEM-1010 transmission electron microscope (Jeol, Japan) equipped with a Morada digital camera (Olympus, Germany).

2.7.3. Differential staining of hemocytes

200 µl of hemolymph were fixed with 20 µl of 5% formalin in PBS. Samples were subsequently placed on a round glass coverslip and kept in the dark for 15 minutes to allow cells to adhere to the glass. Subsequently, samples were air-dried and then incubated with May Grunwald-Giemsa (MGG) quick stain kit (Bio-Optica, Italy) (diluted 1:20 in sterile PBS) for 10 minutes. After three washes with PBS, slides were mounted with Eukitt (Bio-Optica) and analyzed with a Nikon Eclipse Ni-U microscope (Nikon, Japan) equipped with DS-SM-L1 (Nikon) digital camera.

2.7.4. Phagocytosis assay

To investigate the phagocytic activity of plasmatocytes, 5 µl of pHrodo TM Red *Staphylococcus aureus* BioParticles Coniugate (0.2 mg/ml) or pHrodo TM Green *E. coli* BioParticles Coniugate (0.2 mg/ml) (Molecular Probes) were injected in the larvae. 15 minutes, 30 minutes, and 1 hour after the injection, hemolymph was extracted from the larvae. 200 µl of samples were put in a 96 wells plate for 30 minutes in the dark to allow cell adhesion. The supernatant was discarded, cells were washed twice with sterile PBS and resuspended in 200 µl of Schneider's Insect Medium (Sigma-Aldrich). Phagocytosis activity was evaluated using an Olympus IX51 fluorescence microscope (Olympus, Japan) equipped with a DXM1200F digital camera (Nikon).

2.7.5. Encapsulation assay

Spheres of Polymyxin B-Agarose, Sephadex G-100, CM Sephadex C-25, and DEAE Sephadex (Sigma-Aldrich) were used to analyze the encapsulation process. The spheres were washed three times with sterile PBS and resuspended in the same buffer, centrifuging at 1620 x g for 2 minutes at each step. 5 µl of the final preparation were injected into the larvae. The hemolymph was collected at different times (2, 4, 14, and 24 hours) and diluted in a 2:1 ratio (hemolymph/medium) (v/v) with Schneider's Insect Medium (Sigma-Aldrich). The encapsulation process was evaluated by observing the samples with an Olympus IX51 microscope (Olympus) equipped with Optika C-P20M camera (Nikon).

2.8. Analyses of the humoral response

2.8.1. Activity of phenoloxidase system: *in vitro* and *in vivo* assays

The hemolymph extracted from larvae was centrifuged at 250 x g for 5 minutes at 4° C to separate the cellular from the humoral fraction. Both an *in vivo* and an *in vitro* assay were set up to analyze the activation of the proPO system.

For the *in vivo* assay, naïve larvae and larvae injected with increasing concentrations of the *E. coli*/*M. luteus* mix (i.e., 10^5, 10^7, 10^8 and 10^9 CFU/ml) were incubated for 7 minutes at RT. 10 µl of hemolymph were added to 990 µl of 8 mM L-Dopa (L-3-4 dihydroxyphenylalanine) (Sigma-Aldrich) in 10 mM Tris - HCl in a 1.5 ml cuvette.

The activation of the proPO system was evaluated *in vitro* by incubating 10 µl of hemolymph of naïve larvae with 10 µl of Zymosan from *Saccharomyces cerevisiae* (Sigma-Aldrich), or 10 µl of 1 mg/ml trypsin (Sigma-Aldrich) and 980 µL of 8 mM L-Dopa (Sigma-Aldrich) in 10 mM Tris-HCl in a 1.5 ml cuvette.

The relative activity of phenoloxidase (formation of dopacrome) was evaluated as an increase in optical density over time, at 490 nm for 60 minutes, using a V-560 double-beam spectrophotometer (Jasco, USA).

2.8.2. Lysozyme activity

Larvae were injected and hemolymph extracted after 6, 14, 24, and 48 hours (see "Injection of larvae and collection of hemolymph" paragraph). N-Phenylthiourea (PTU, Sigma-Aldrich) was added to the hemolymph to avoid the activation of PO system. Samples were centrifuged twice at 250 x g for 5 minutes at 4 °C, then once at 1600 x g for 10 minutes at 4 °C, and finally diluted 1:10 with sterile PBS. *M. luteus* (0.45 mg/ml in 30 mM phosphate buffer, pH 7.2; Sigma-Aldrich), with an optical density of 0.6-0.7 was used as substrate. 100 µl of hemolymph samples were added to 150 µl of bacterial suspension. *M. luteus* without hemolymph and hemolymph without addition of the bacterium were used as controls. Absorbance at 450 nm was measured every 30 s for 10 min, in a 96 well-plates using a Bio Rad iMark™ Microplate Absorbance reader (Bio-Rad, USA).

2.8.3. qRT-PCR

After the infection, control and immunized larvae were anesthetized on ice and then dissected. Fat body was isolated, frozen in liquid nitrogen, and stored at -80 °C until use. RNA was extracted from 30-40 mg of tissue with Trizol Reagent (Life Technologies, USA) and genomic DNA contamination was removed by TURBO DNA-free Kit (Life Technologies). RNA quality was verified through gel electrophoresis.

Retrotranscription was performed with M-MLV reverse transcriptase (Life Technologies). Primers used for qRT-PCR are listed in Table 1. RPL5 was used

as housekeeping gene. Real-Time PCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad), including for each reaction mix the control template (NTC) in which water was added instead of cDNA. A CFX Connect Real-Time PCR Detection System (Bio-Rad) was used for cycling conditions (95 °C for 30 s, followed by 39 cycles of 95 °C for 10 s, and 60 °C for 30 s). Relative expression of the genes was calculated with the $2^{-\Delta\Delta Ct}$ method. Each value was the result of experiments performed on five series of samples.

Table 1. Sequence of primers used in this study

Gene name	Trascriptome Accession number	Contig number	Primer sequences
HiDefensin	ERP122672	TRINITY_DN10226_ C0_G1_I2	F: GCGTTCTATTCTCGTCTTGG
			R: TGCTGTTCCACTACCTGACT
HiDiptericin	ERP122672	TRINITY_DN6246_ C0_GI_I1	F: CCCAGTGAGCGATGAGGAA
			R: GTGAAGGGTATTGCGTCCAT
HiLysozyme	ERP122672	TRINITY_DN12175_ C0_G2_I1	F: GCCCAAGGCAAGGTTTACA
			R: TGGCGAGGGTGGTTAGATTC
HiRPL5	ERP122672	TRINITY_DN8551_ C0_G1_I2	F: AGTCAGTCTTTCCCTCACGA
			R: GCGTCAACTCGGATGCTA

2.9. Statistical analysis

ANOVA followed by Tukey's HSD test (significance $p<0.05$) were performed for the statistical analysis.

3. Results

3.1. Determination of the bacterial concentration

The bacterial concentration used in the analyses was determined monitoring larvae injected with different concentrations of an *E. coli*/*M. luteus* mix (from 10^4 to 10^9 CFU/ml) up to 72 hours. As shown in Figure 1, the survival of the larvae decreased proportionally with the bacterial dose administered. In fact, while 100% of larvae were alive when injected with a 10^4 CFU/ml concentration, after the infection with higher bacterial doses (10^5, 10^6, 10^7 and 10^8 CFU/ml) the percentage of live insects proportionally decreased (72%, 64%, 60% and 16% respectively). All the animals died in 72 hours at the highest concentration tested (10^9 CFU/ml) (Fig. 1).

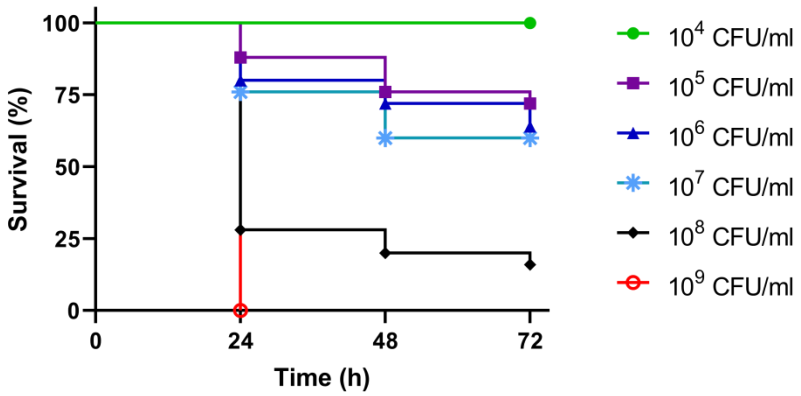

Figure 1. Determination of the bacterial concentration. Survival rate of larvae infected with different concentrations of *E. coli*/*M. luteus* mix

These experiments allowed us to establish the concentration needed to reduce the welfare of the larvae and stimulate the immune system, without causing an excessive mortality of the insect in order to monitor the immune response over time. Therefore, all the analyses were performed by injecting 10^5 CFU/ml of the bacterial mix into the larvae.

3.2. Analysis of the antimicrobial activity

We first evaluated the antimicrobial activity of the hemolymph. For this reason, an *in vitro* and an *in vivo* assay were set up to define the role of the humoral response and to evaluate the combined response of both immune components, respectively.

In vitro assay

The antimicrobial activity against *E. coli* and *M. luteus* was evaluated by removing hemocytes from the hemolymph. The results showed a time-dependent trend of the antimicrobial activity of the humoral component of the hemolymph against *E. coli*. In fact, while bacterial concentration in naïve larvae was 2.26×10^9 CFU/ml, it dropped to 1.16×10^6 CFU/ml and 3.08×10^4 CFU/ml 6 and 14 hours after the infection, respectively. *E. coli* concentration dropped to 0 CFU/ml within 48 hours (Fig. 2A). On the contrary, the antimicrobial activity tested against *M. luteus* showed a different trend. In fact the initial concentration of *M. luteus* in naïve larvae was 5.77×10^9 CFU/ml, while it decreased to 1.5×10^9 CFU/ml after 6 hours. The values remained almost constant after 14, 24, and 48 hours (Fig. 2B).

In vivo assay

The *in vivo* assay was performed by injecting *H. illucens* with *E. coli* or *M. luteus* and plating the hemolymph on LB agar plates.

E. coli concentration ($5{,}73 \times 10^2$ CFU/ml after 6 hours) dropped to zero only 14 hours after the infection (Fig. 2C). Conversely, *M. luteus* grew progressively from 6 up to 14 hours (4.34×10^5 CFU and 2.62×10^6 CFU, respectively), but no CFU were detected within 24 hours from the infection (Fig. 2D).

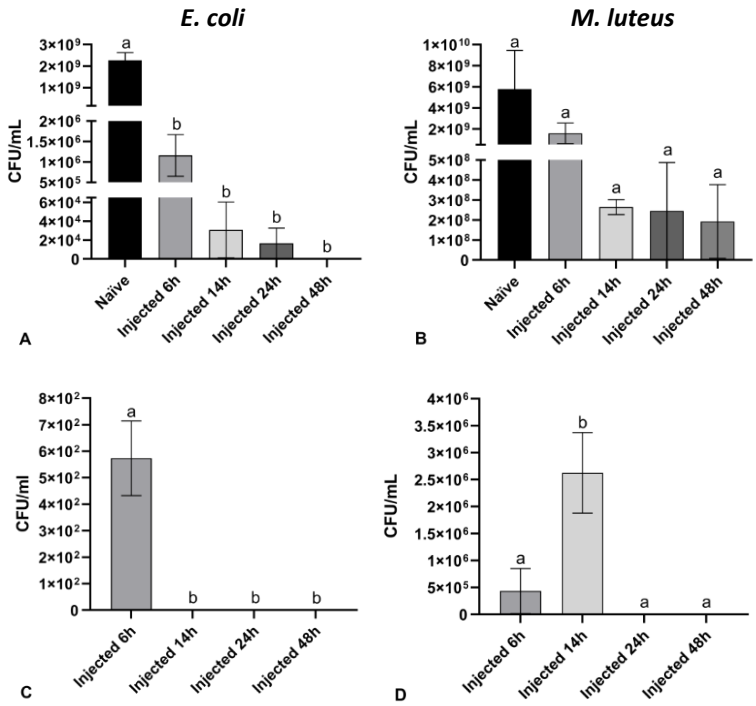

Figura 2. Analyses of the antimicrobial activity. A, B) *In vitro* assay against *E. coli* (A) and *M. luteus* (B). **C, D)** *In vivo* assay against *E. coli* (C) and *M. luteus* (D). Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

3.3. Cellular immune response

3.3.1. Total hemocyte count

To quantify the cells present in the hemolymph of the larvae and to evaluate variations in their number after infection, total hemocyte counts were performed. The quantification of the cells highlighted a significant difference between the cell number of naïve (8,45 x 10^5 cells/ml) and infected larvae (2,19 x 10^6 cells/ml) after 6 hours (Fig. 3A). The difference between control and infected larvae was stable up to 24 hours (4,59 x 10^5 cells/ml and 2,03 x 10^6 cells/ml, respectively), while it was not observed 48 hours after the injection (6.43 x 10^5 cells/ml in naïve larvae; 1,55 x 10^6 cells/mL in injected larvae) (Fig. 3A). The puncture with sterile needle and the injection with sterile PBS did not affect the immune response of

the insect, in fact no significant differences were present between these control groups and naïve larvae (Supplementary materials, Fig. S1).

The immune response of the larvae can also be affected by the environment in which the larvae live. For this reason we performed an immune challenge and quantified the hemocytes isolated from animals reared on non-sterile diet. A significant increment of the cell number was observed starting from 14 hours after the injection (2.73 x 10^7cells/ml). This data remained stable up to 48 hours (2.35 x 10^7 cell/ml) (Fig. 3B).

These analyses demonstrate the great involvement of hemocytes following the activation of the immune response by pathogens.

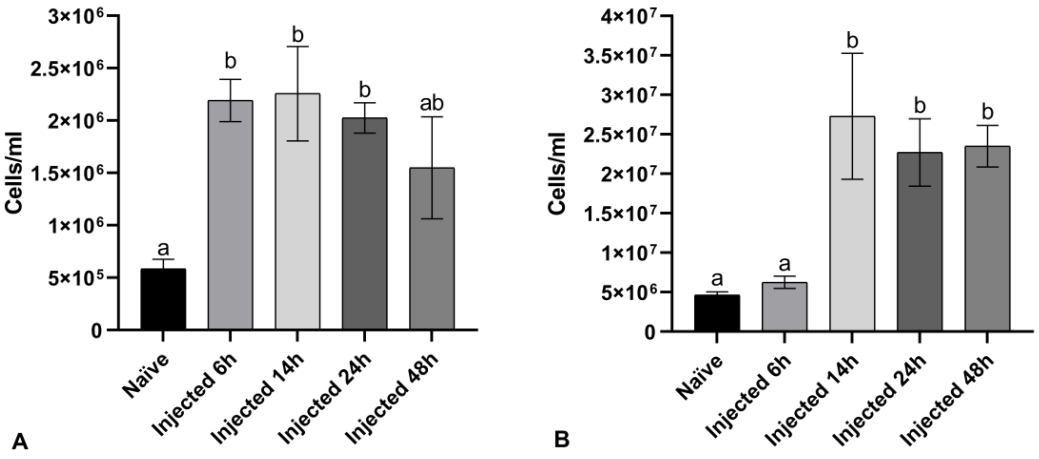

Figure 3. Total count of hemocyte of starved (A) and fed larvae (B). Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

3.3.2. Differential cell count and characterization of hemocytes

The morphological analysis evidenced the presence of five different types of hemocytes. In particular, plasmatocytes were the most abundant cells in the hemolymph of *H. illucens* larvae (about 90% of the total). A smaller fraction of hemocytes (7%) was represented by prohemocytes, small round cells with a high nucleus:cytoplasm ratio (Fig. 4A,B). Finally the remaining cell types identified (i.e., lamellocytes, crystal cells, and granulocytes) represented a fraction of less than 1%.

More in detail, plasmatocytes possessed a central lobed nucleus and a basophilic cytoplasm (Fig. 4C). TEM analysis revealed the presence of abundant lysosomes and a developed rough endoplasmic reticulum; moreover several plasma membrane expansions were visible (Fig. 4D,E). Lamellocytes possessed a round or oval nucleus and they appeared elongated with apical expansions (Fig. 4F,G). The non-adherent crystal cells were oval-shaped hemocytes characterized by several crystal inclusions (Fig. 4H-L). Finally, granulocytes had several basophilic and acidophilic granules (about 0.4 μm) in the cytoplasm and a lobed nucleus (Fig. 4M-O).

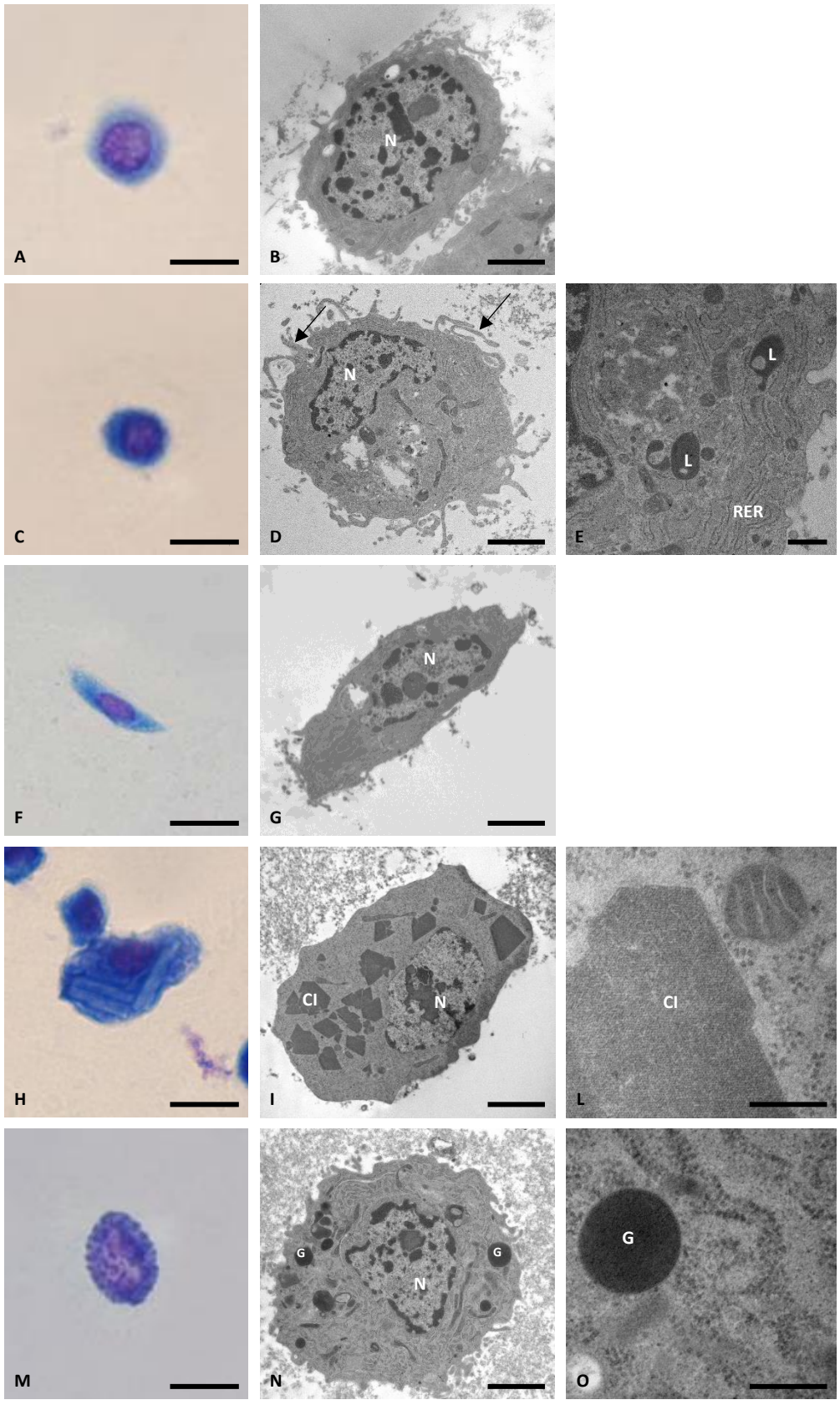
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Figure 4. Morphological characterization of hemocytes. A, B) Prohemocyte; **C-E)** Plasmatocyte; arrows: pseudopods; **E)** TEM detail of a plasmatocyte; L: lysosomes; RER: rough endoplasmic reticulum; **F, G)** Lamellocyte; **H-L)** Crystal cell; **L)** TEM detail of a crystal cell; CI: crystal inclusion; **M-O)** Granulocyte; **O)** TEM detail of a granulocyte; G: cytoplasmic granule. N: nucleus; Bars: 10 μm (A, C, F, H, M); 2 μm (B, D, G, I, N); 200 nm (E, L, O).

3.3.3. Phagocytosis

To analyze phagocytosis, we injected Gram-positive (*Staphylococcus aureus*) and Gram-negative (*E.coli*) bacteria conjugated with the pHrodo fluorophore in the larvae. In both cases, we observed a time-dependent increase in phagocytosis activity. In particular, while very few cells able to engulf the fluorescent bacteria were detected 15 and 30 minutes after infection (Fig. 5A-D), an increased quantity of cells was observed after 1 hour (Fig. 5E-F).

These data demonstrate that phagocytosis is rapidly activated after pathogen recognition.

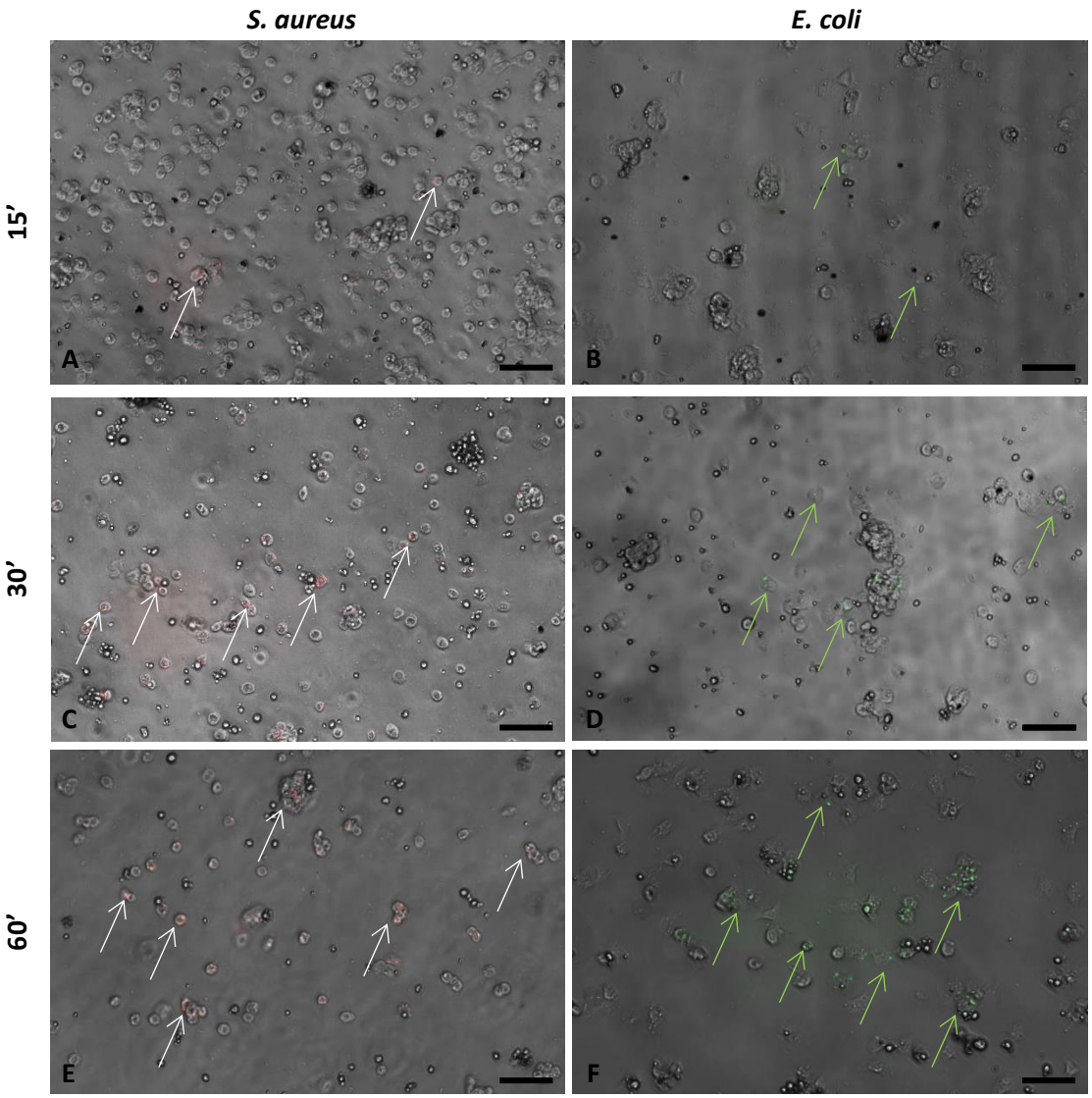

Figure 5. Phagocytosis. Hemocytes observed 15 (A,E), 30 (B,F), and 60 minutes (C,G) after the injection of *S. aureus* (A-D) and *E. coli* (E-H) conjugated with the pHrodo fluorophore. Bars: 40 μm.

3.3.4. Encapsulation process

The kinetics of activation of the encapsulation process was analyzed through the injection of different types of beads.

We first compared the effects of neutral charged agarose and dextran spheres (Fig. 6). In particular, in larvae treated with Polymyxin B-Agarose spheres

encapsulation initiated 2 hours after the administration (Fig. 6A). The number of cells adhering to the spheres significantly increased within 4 hours (Fig. 6B). Despite the massive recruitment of hemocytes at 14 and 24 hours after the infection (Fig. 6C,D), the production of melanin was never observed. Conversely, when Sephadex G-100 spheres were injected in the larva (Fig. 6E-H), the amount of adherent hemocytes to the beads was reduced and melanin deposition was found at 14 and 24 hours (Fig. 6G,H).

Secondly, we focused our attention on Sephadex matrix, and we used negative and positive-charged beads to understand the effect that different charges can have on the encapsulation process and melanin deposition. In particular, in larvae treated with CM Sephadex C-25 spheres (negatively charged), hemocytes began to adhere to the spheres very quickly (2-4 hours from the injection in the larva) (Fig. 7A,B). Although the amount of haemocytes did not increase at longer times (Fig. 7C,D), 24 hours after the injection the spheres were completely melanized (Fig. 7D). Conversely, the injection of the larvae with DEAE Sephadex spheres (positively charged) determined a complete melanization of the *non-self* agent earlier (4 hours) (Fig. 7F). Cells, which were closely attached to the spheres even at the shortest times (Fig. 7E,F), formed a typical melanotic capsule within 24 hours (Fig. 7G,H).

Summarizing we can conclude that the matrix and the charge of the sphere can significantly affect the encapsulation process.

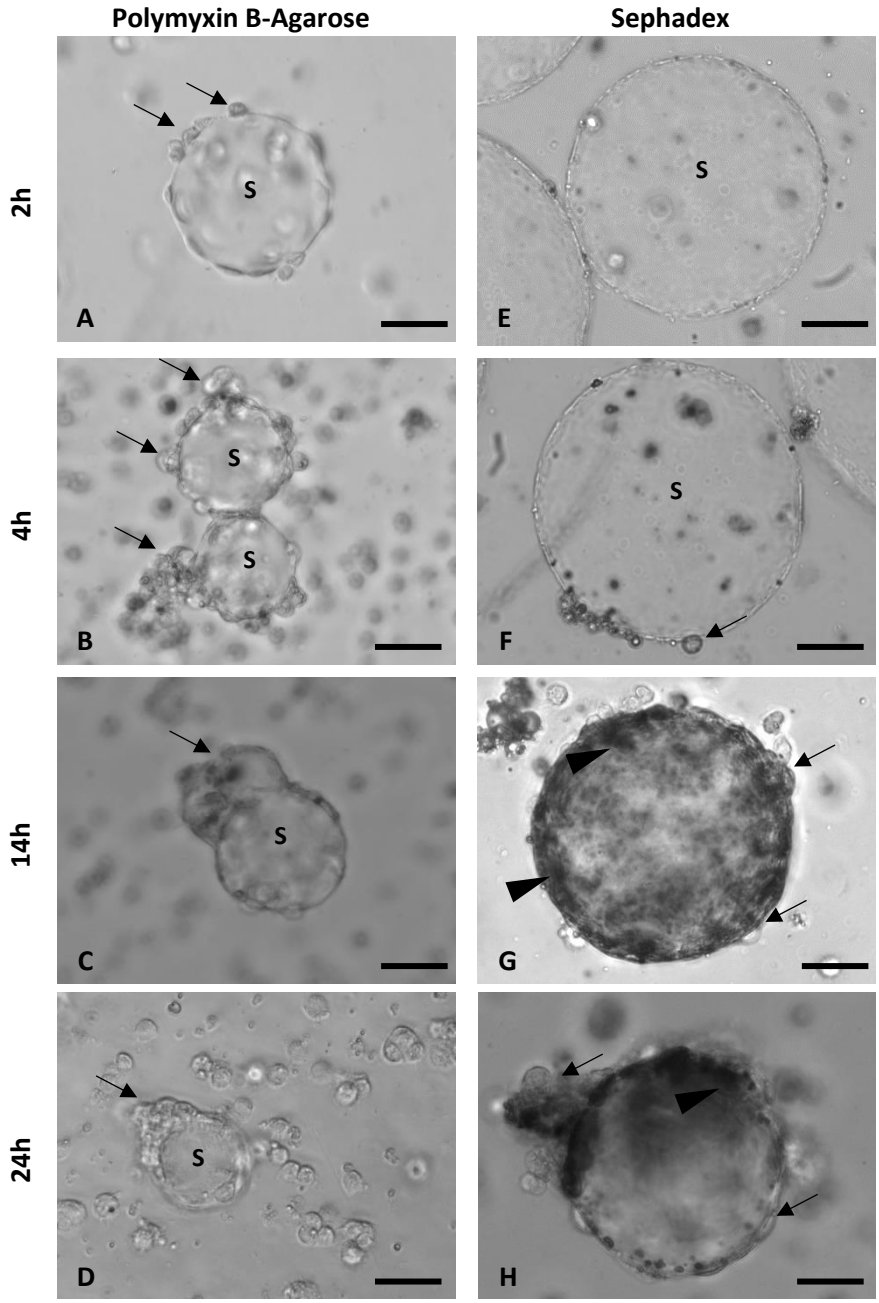

Figure 6. Encapsulation assay with Polymyxin B-Agarose (A-D) and Sephadex spheres (E-H). Hemolymph was analyzed after 2 (A,E), 4 (B,F), 14 (C,G), and 24 hours (D,H) from the injection. Arrows: cells adherent to the spheres; Arrowheads: melanin deposition; S: spheres. Bars: 40 μm.

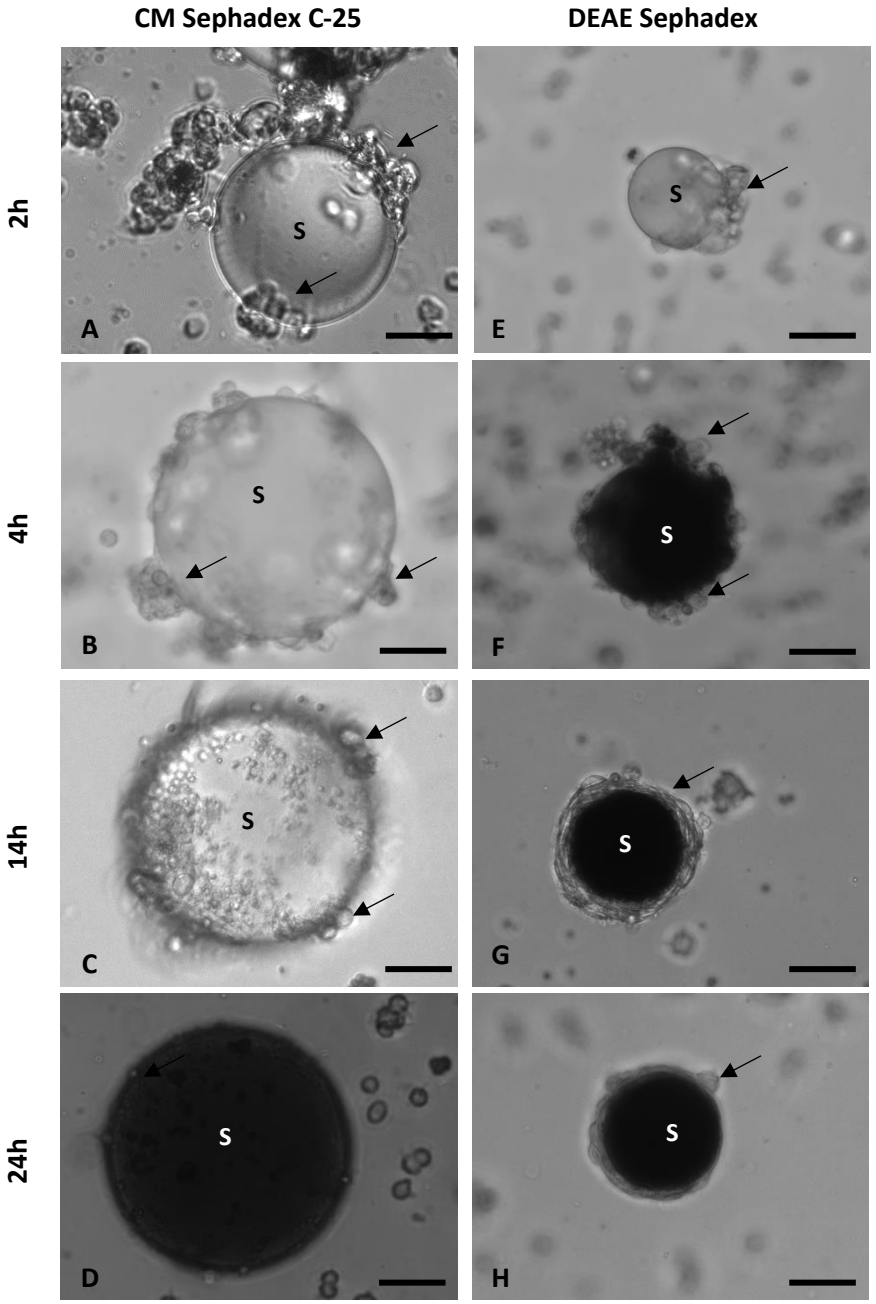

Figure 7. Encapsulation assay with CM Sephadex C-25 (A-D) and DEAE Sephadex spheres (E-H). Hemolymph was analyzed after 2 (A,E), 4 (B,F), 14 (C,G), and 24 hours (D,H) from the injection. Arrows: cells adherent to the spheres; S: spheres. Bars: 40 μm.

3.4. Humoral immune response

3.4.1. proPO system

The activation of the proPO system was initially monitored in naïve larvae and larvae injected with 10^5 CFU/ml bacterial mix. While in naïve larvae the absorbance increased up to 60 minutes, an inhibition of the enzymatic system was observed following bacterial infection. In this case, the absorbance remained stable over time (Fig. 8).

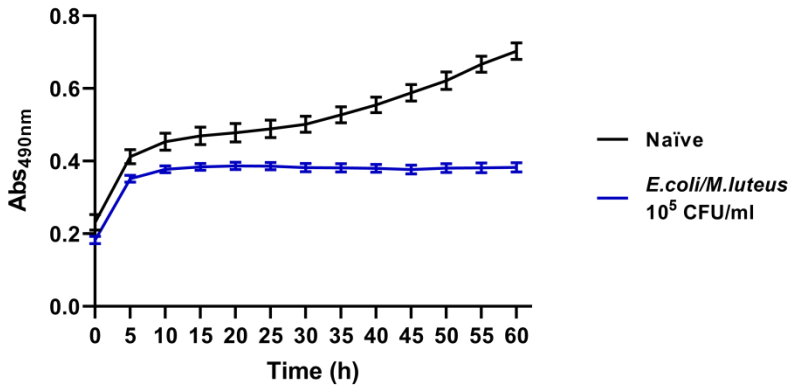

Figure 8. proPO system. Comparison between hemolymph of naïve larvae and larvae injected with 10^5 CFU/ml of *E.coli*/*M. luteus* mix.

Two different system activators (i.e., trypsin and Zymosan) were added to the hemolymph samples to verify the functionality of the phenoloxidase system in naïve larvae (Fig. 9). The results showed a consistent and rapid activation of the phenoloxidase system when stimulated with trypsin: the absorbance in fact increased exponentially over time reaching the maximum threshold (i.e., 1.00) after 40 minutes. Although a slower increase in the enzyme activity in the first 15 minutes was observed after the stimulation with Zymosan, similarly to the previous condition the absorbance reached the threshold value within 40 minutes from the beginning (Fig. 9).

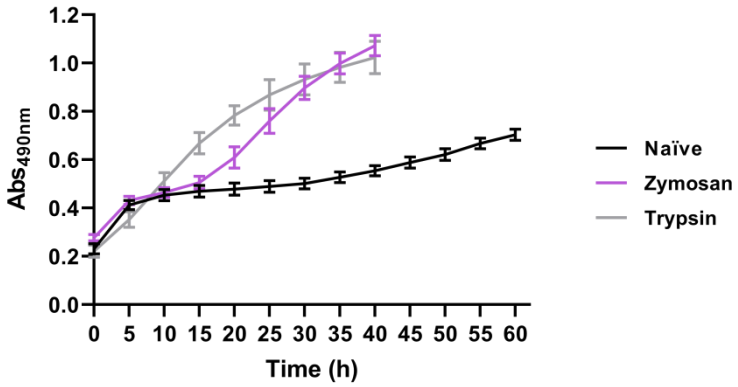

Figure 9. proPO system. Comparison between hemolymph of naïve larvae and hemolymph added with trypsin or Zymosan.

Once the correct functionality of the enzymatic system in naïve larvae was demonstrated, a possible dependence of the proPO activation on the bacterial concentration was evaluated. To this purpose, the activity of the phenoloxidase system in larvae injected with a bacterial mix at 10^5, 10^7, 10^8, and 10^9 CFU/ml was compared (Fig. 10). The data obtained showed the inhibition of the system by the bacterial mix at a concentration lower than or equal to 10^7 CFU/ml (Fig. 10). A different trend of the absorbance was visible after the injection of 10^8 CFU/ml, which represented the threshold concentration at which an activation of the enzyme complex was observed.

These results allowed us to conclude that the proPO system is activated by the larvae only in the presence of a high bacterial load in the hemolymph.

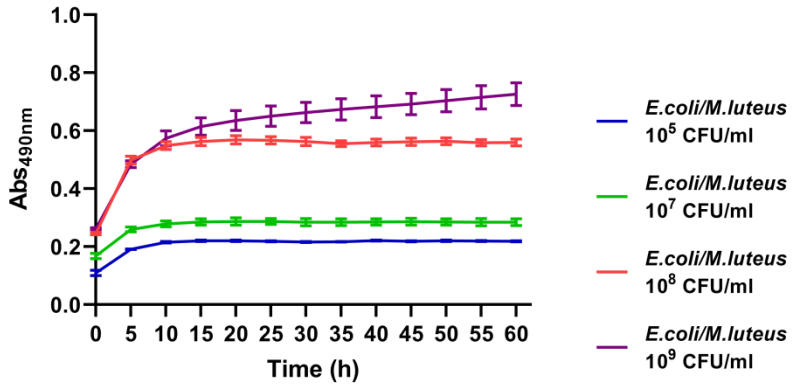

Figure 10. ProPO system. Comparison between hemolymph of injected larvae with different concentrations of *E.coli/M.luteus* bacterial mix.

3.4.2. Expression and activity of Lysozyme

Thanks to its muramidase activity, lysozyme is able to cleave the cell wall of Gram-positive bacteria bringing them to death. The analysis of the transcription levels of the gene coding for lysozyme, performed in starved larvae, did not detect any significant difference in the mRNA levels between control group and injected larvae up to 14 hours. Interestingly, a significant decrease of the mRNA expression was observed at 24 hours (Fig. 11A). The negative control performed by injecting the larvae with sterile PBS or puncturing them with a sterile needle did not show any increase of lysozyme mRNA levels (Supplementary materials, Fig. S2).

On the contrary, the levels of lysozyme expression markedly increased when larvae were reared within the feeding diet after the bacterial injection (Fig. 11B). In particular, 24 hours after the infection a 40-fold increase of lysozyme expression compared to control group was observed (Fig. 11B).

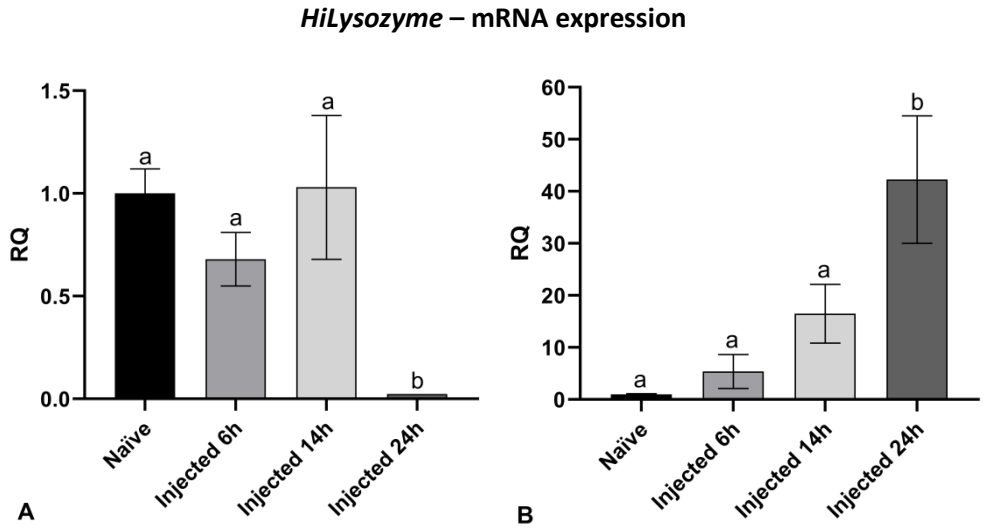

Figure 11. qRT-PCR analysis of *HiLysozyme*. mRNA levels in starved larvae (A) and in fed larvae (B). Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

Subsequently, we analyzed lysozyme activity in the hemolymph. In starved animals, a basal activity of the enzyme was observed in naïve larvae, while it progressively increased in infected larvae (Fig. 12). In detail, starting from 6633 ± 133 U/ml after 6 hours, it increased up to 9100 ± 900 U/ml after 14 and to 11316 ± 687 U/ml at 24 hours. Finally it reached the maximum value 48 hours post-infection (25633 ± 2338 U/ml) (Fig. 12). The negative control did not show any activation of the enzyme, demonstrating the inability of the buffer to trigger the immune response of the insect (Supplementary materials, Fig. S3).

As for the total hemocyte count, we tested the lysozyme activity in fed larvae, but no difference in the activity was observed compared to starved animals (data not shown).

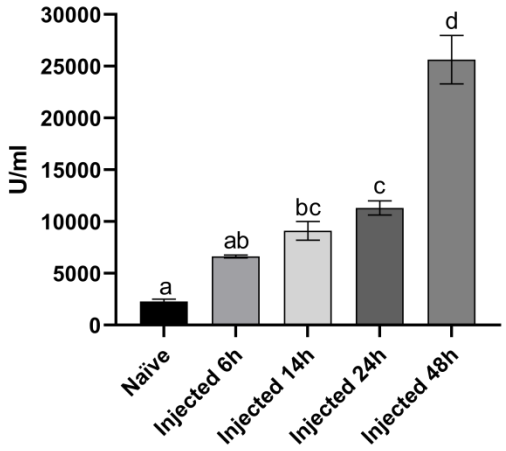

Figure 12. Lysozyme activity in starved insects: comparison between hemolymph of naïve and injected larvae

3.4.3. Antimicrobial peptides expression

AMPs are rapidly produced to counteract bacterial infection. Herein, we monitored mRNA transcription of genes coding for Defensin (active against Gram-positive bacteria) and Diptericin (active against Gram-negative bacteria) on starved and fed animals.

In starved animals, the mRNA levels of both AMPs did not vary significantly within 3 hours from the infection. An expression peak of Diptericin was observed at 6-14 hours after the infection, then mRNA levels returned to baseline values within 24 hours (Fig. 13A). Defensin expression instead increased only after 14 hours and remained constant up to 24 hours (Fig. 13B). As concern Diptericin, no difference was found in expression levels in larvae that were punctured with the needle or treated with PBS compared to naïve larvae (Supplementary materials, Fig. S4A). Contrarily, the expression levels of Defensin in larvae injected with sterile PBS showed a significant difference in comparison to naïve larvae and

larvae punctured, although these levels were very different compared to infected larvae (Supplementary materials, Fig. S4B).

Interestingly, the expression of both genes in infected animals kept on the diet was higher compared to those of starved animals (Fig. 13C,D). Moreover, while Diptericin expression peaked at 6 hours and then the mRNA levels decreased after 14 hours post-injection (Fig. 13C), the expression of Defensin remained at high levels 6-14 hours after the injection of bacteria and then returned to basal levels after 24 hours (Fig. 13D).

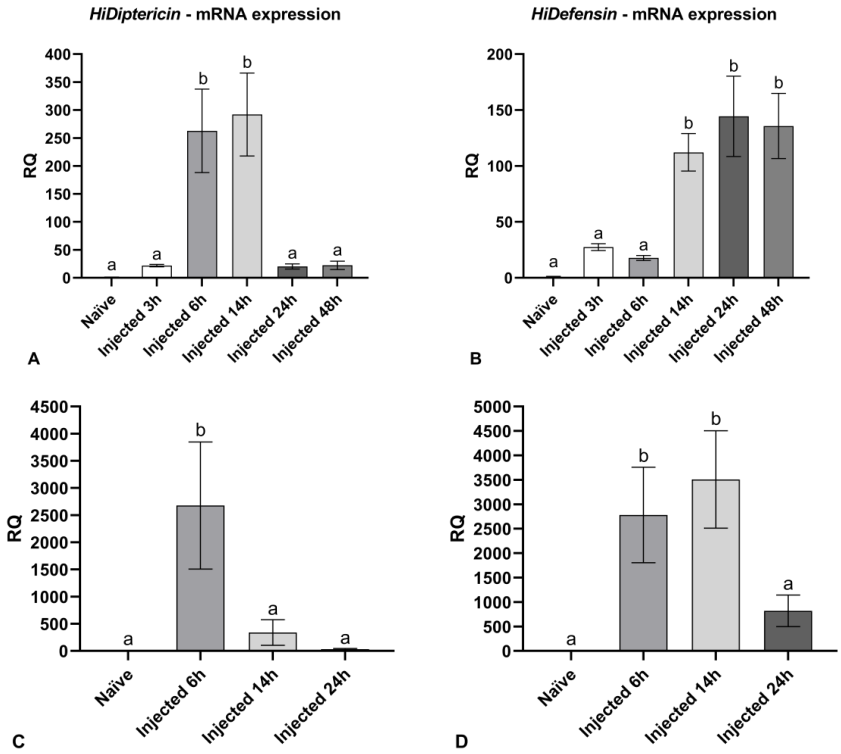

Figure 13. qRT-PCR analysis of *HiDiptericin* (A,C) and *HiDefensin* (B,D). mRNA levels in starved (A,B) and fed larvae (C,D). Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

4. Discussion

Although the interest toward *Hermetia illucens* is exponentially increasing and different papers about the biology of this insect are available (Bonelli et al., 2019; Bonelli et al., 2020; Bruno et al., 2019a; Pimentel et al., 2017), information regarding immunity still remains fragmentary. To date only few papers are present in the literature, which mainly focus on the humoral response, and in particular on the characterization of AMPs (Zdybicka-Barabas et al; 2017; Elhag et al., 2017; Vogel et al., 2018). The immune challenge conducted in this study allowed us to investigate the responses of the larvae following the damage of the cuticle, due to the injection, and following the administration of a precise concentration of a bacterial mix. For this reason we considered naïve larvae, i.e. completely undamaged, as our reference control. Moreover, we carried out all the analyses keeping the larvae in a sterile condition, and therefore in starvation, to avoid that the injection and the possible pathogens present in the surrounding environment could cause a double infection to the insects. In fact the analyses conducted in parallel using a pathogen-free diet (Bruno et al., 2019b) aimed at evaluating how the rearing procedures could affect the immune response, showed a marked increment of the markers tested. This is probably caused by a "super-infection" of the larvae, due both to the injection of bacteria and to the exposure to the surrounding environment. Therefore we can hypothesize that the immune response undergoes more pronounced modifications when the larvae are in presence of a diet contaminated by entomopathogens, as in the case of mass rearing procedures. These results also highlight the high plasticity of the immune system, which is subjected to continuous changes in relation to the environment.

To overcome the lack of information regarding the cellular response of *H. illucens*, we firstly characterized the hemocytes of this insect. The three main types of haemocytes, i.e. plasmatocytes, crystal cells and lamellocytes, also described in other Diptera as *Drosophila* (Lavine and Strand, 2002), were observed confirming evidence obtained in the only study available on the immune system of *H. illucens* larvae (Zdybicka-Barabas et al., 2017). In addition, several granulocytes were found in the hemolymph, which are important for the initial recognition of pathogens and for the encapsulation of large foreign targets (Lavine and Strand, 2001; Zdybicka-Barabas et al., 2017). We observed that the timing of activation of the process in which these cells are involved (i.e., phagocytosis, encapsulation) is very short. In fact, the assay performed with pHrodo-conjugated bacteria showed how plasmatocytes have a high efficiency and rapidity in phagocytizing the pathogens as described for other insects (Hillyer et al., 2003), acting just after 15 minutes from the infection, but differently from what reported by Hernández-Martínez and colleagues for *Anopheles albimanus* (2002), we did not highlight differences among the type of bacteria injected. The dynamic of encapsulation is slightly different: at least two hours are needed to observe cells involved in this process and more time for the formation of a melanotic capsule. Moreover, as described in other insect orders (Lackie, 1983; Gotz and Vey, 1986; Gorman et al., 1998; Lavine and Strand, 2001), it should be emphasized how the matrix composition and the charge of the chromatography beads used in this study determine a different affinity of the cells and affect the entire process of the *non-self* encapsulation. In fact, although agarose beads are recognized and bound by cells, which also form the capsule, they are not subjected to melanization, as demonstrated in other insects (Gorman et al., 1998; Lavine and Strand; 2001). This result is different for the dextran spheres, which at the end of the experiment are completed melanized and

encapsulated. As demonstrated by Gorman and colleagues (1998) the melanotic encapsulation is induced by glucans, which activate the proPO enzymatic cascade and which are present in this kind of spheres. Moreover, we demonstrated that also the charge of the spheres induced a different degree of melanization: the greatest deposition of melanin occurs in the presence of positive charges, as also observed in other insect orders, especially in Diptera and Lepidoptera (Paskewitz and Riehle, 1994; Lavine and Strand, 2001)

Also the time required to encapsulate the foreign targets varies: in fact both CM- and DEAE-Sephadex beads were rapidly encapsulated, while it took much longer time for neutral beads. As reported by Lavine and Strand (2001), this could probably be due to the recognition by granular cells: in particular, spheres that are encapsulated in short times are quickly recognized by the PRRs present on these hemocytes, while spheres that are encapsulated more slowly must undergo opsonization by the same receptors before being subjected to encapsulation (Lavine and Strand, 2001). Further investigations are surely needed to confirm this hypothesis and establish how plasmatocytes, granulocytes, and crystal cells intervene to form the melanotic capsule around the foreign targets.

To trigger an adequate cellular response for counteracting the bacterial infection, the number of hemocytes increases quickly after the injection of bacteria. This is plausibly due to proliferation phenomena and/or to the recruitment of prohemocytes and subsequent differentiation (Lanot et al., 2001). It is interesting to note how, after the initial increase in haemocytes in infected larvae, the number of these cells remains stable, suggesting that the amount of cells produced during the first hours after the infection is sufficient to control the bacterial infection.

Differently to the activity of the hemocytes, the induction of the humoral response requires more time, in accordance to the literature (Lavine et al., 2005; Haine et al., 2008). Although basal levels of lysozyme are present in fat body cells of healthy larvae (Dunn et al., 1985), its activity increases several hours after the infection, and its expression is maintained also after longer time (24 and 48 hours). As suggested by Boman et al. (1972), this is probably necessary to avoid a possible second infection. Moreover, lysozyme could act as a negative regulator of the proPO system: it is maintained active while a proPO inhibition is observed, as indicated also by Zdybicka-Barabas et al. (2014). As concern the phenoloxydase system, our results demonstrate that this enzyme is not activated in the case of minor bacterial infections for which cellular process, antimicrobial peptides, and lysozyme are sufficient (Imler and Bulet, 2005; Melcarne et al., 2019). In fact, proPO is activated only by high doses of bacteria (10^9 CFU/ml), confirming the observations conducted on *G. mellonella* (Pye, 1978) and *B. mori* (Montali et al., 2020). This could be explained considering the toxicity of melanin and molecules produced during the enzymatic cascade. It is plausible that other immune components are activated by the larvae before triggering the proPO system to counteract the infection, avoiding its excessive and/or uncontrolled activation which could affect the health of the larva (Lemaitre and Hoffmann, 2007; An et al., 2011; Lu et al., 2014).

It is also important to highlight how, differently to the cellular components, the action of the humoral fraction against Gram-negative and Gram-positive bacteria is different. In fact, Diptericin expression levels are drastically reduced after 24 hours from the infection, indicating that transcripts produced within 14 hours are sufficient to produce a quantity of AMP able to contain the bacterial infection, while Defensin mRNA levels remain high up to 24 hours, suggesting how the control of Gram-positive bacteria proliferation requires more time. The evidence

on the antimicrobial activity of the humoral component corroborated these results. In fact, while the only humoral fraction has a bactericidal effect on *E. coli*, it is effectively unable to stop *M. luteus* growth despite the continued production of molecules with Gram-positive antimicrobial activity, such as Defensin or lysozyme. On the contrary, analyses performed on hemolymph containing both the humoral and cellular fractions showed total removal of both bacteria, demonstrating the importance of a synergic action of the two components of the immune response (Elrod-Erickson et al., 2000).

In conclusion, this study allowed to obtain an in-depth characterization of the *H. illucens* larvae immunity and highlighted for the first time the different timing of activation of the mechanisms involved. Moreover, it not only provides important knowledge on the functionality of *H. illucens* immune system, but it also lays the basis for future studies on its possible modulation through external environmental factors, such as diet and temperature, two important aspects in mass rearing.

5. Acknowledgement

A.M. is a Ph.D. student of the 'Life Science and Biotechnology' course at University of Insubria. The authors wish to thank Mario Grassi for technical support.

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Supplementary materials

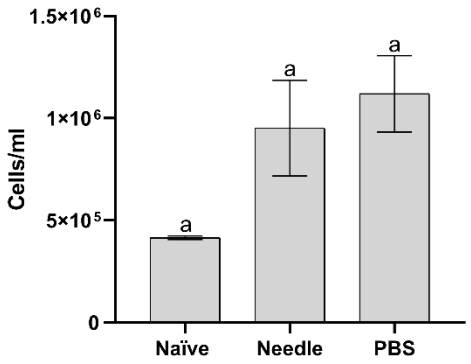

Figure S1. Total count of hemocyte: comparison between naïve larvae (uninjected), punctured with a sterile needle and injected with sterile PBS. Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

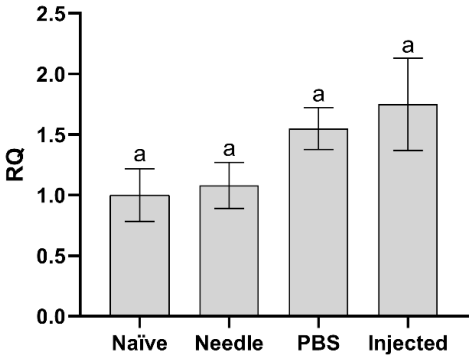

Figure S2. qRT-PCR analysis of *HiLysozyme*. mRNA levels of *HiLysozyme* in samples of naïve larvae, punctured with a sterile needle (Needle) and injection of sterile 1X PBS (PBS) compared to infected (Injected) larvae after 14 hours from starvation. Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

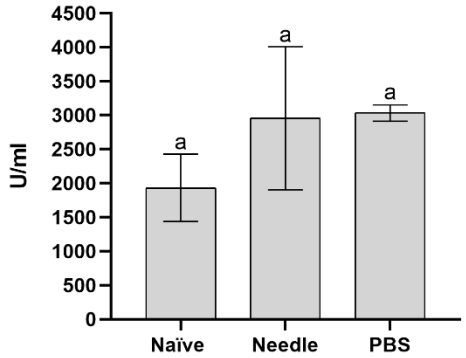

Figure S3. Comparison of lysozyme activity tested on hemolymph of naïve larvae (uninjected), punctured with a sterile needle and injected with sterile PBS. Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

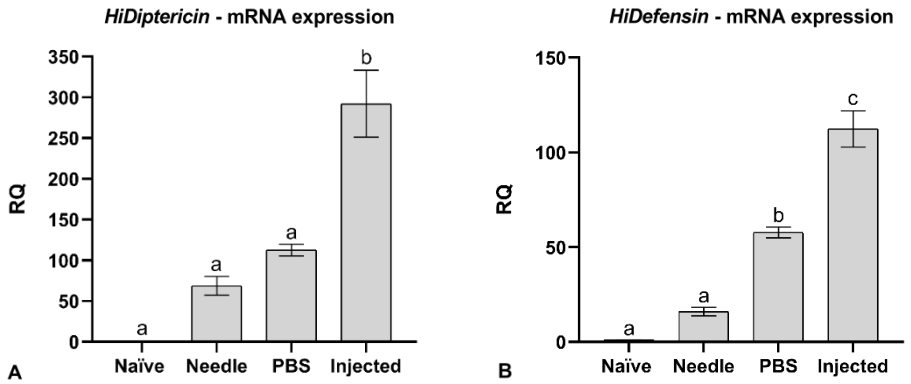

Figure S4. qRT-PCR analysis of AMPs. mRNA levels of *HiDipetericn* (A) and *HiDefensin* (B) in samples of naïve larvae, punctured with a sterile needle (Needle) and injection of sterile 1X PBS (PBS) compared to infected (Injected) larvae after 14 hours from starvation. Values represent mean ± s.e.m. Different letters indicate statistically significant differences among treatments ($p < 0.05$).

CONCLUDING REMARKS

In this thesis two different biotechnological approaches based on insect immune system were considered. The insect models used in this PhD project differ from different viewpoints. However, both insects are of key importance for the development of biotechnological applications. The lepidopteron *B. mori* plays a fundamental role in sericulture and textile industry and has been extensively used in life sciences studies. Ihe information available in literature has significantly increased over the years, making the silkworm a reference model for biological research. The development of the infection model described in the first chapter of this thesis took benefit from the possibility of having perfectly synchronous larvae from stock centers, unlike many other insects. Conversely, to date, this would not be possible with *H. illucens*, which instead has been increasingly gaining importance only recently for its great ability to convert organic waste and it is not excluded that, due to the continuous research, *H. illucens* will became a widely-used model as *B. mori*.

To briefly summarize, the principal achievements of our research are:

1. the establishment of an invertebrate infection model for the screening of new antimicrobial compounds. The infection of *B. mori* larvae using a severe pathogen for human health (i.e., *S. aureus*), the treatment with glycopeptides antibiotics, and the subsequent analyses of cellular and humoral markers led to develop a protocol that can be used for applicative purposes.

   Our insect infection model does not claim to eliminate the use of mammalian animal models, but it could be included in preclinical tests

before the use of mice to facilitate and speed up the discovery of new antimicrobial drugs.

2. an in-depth characterization of the immune response of *H. illucens* larvae subjected to microbial infection. In particular, the identification of the hemocytes in BSF larvae, which are directly involved in phagocytosis and encapsulation, was performed. These mechanisms are activated early after the recognition of the pathogens, unlike humoral responses. We thus demonstrated a different timing of activation of the two different immune responses. We also observed that the immune response of the larvae is markedly activated when they are reared in a non-sterile environment, and these results can open in the future the possibility of modulating insect resistance to pathogens, an aspect of considerable importance in mass rearing activities. Overall, this study allowed us to fill a significant gap of knowledge on *H. illucens*. In fact, although different studies on the rearing methods and the biology of this insect are available, the information about its immune system is still scarce and fragmentary.