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# The black soldier fly, *Hermetia illucens* – a promising source for sustainable production of proteins, lipids and bioactive substances

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**Abstract:** The growing demand worldwide for proteins and lipids cannot be met by the intensive use of agricultural land currently available. Insect mass cultures as a source for proteins and lipids have been in focus for various reasons. An insect with many positive properties is the black soldier fly, *Hermetia illucens*, whose larvae could be used for the sustainable production of proteins and lipids. Furthermore, the larvae produce bioactive substances which could potentially be used for human and animal welfare.

**Keywords:** antimicrobial peptides; *Bacillus subtilis*; biofilms; *Hermetia illucens*; insect mass culture; insect protein and lipid.

## 1 How could insects help to reduce the worldwide protein and lipid gap?

The worldwide growing demand for proteins and lipids cannot be met by the intensive use of the agricultural land available at present. The growing demand for proteins and lipids has led to the destruction of valuable ecosystems worldwide. The problems are highlighted by the ecological problems due to the expansion of soy production in South America and the booming palm oil production in tropical Asian countries. Slash and burn clearance of tropical forests has been in focus for many years but despite this very unsatisfactory situation a sustainable alternative production for proteins and lipids has not been realized. Because of the ecological limitations the production will increasingly lag behind the demand for proteins (protein gap) and lipids (lipid gap). Is it possible to produce this valuable organic raw material without the use of land? Insect mass cultures as a source for proteins and lipids have become the focus of attention for good reasons. The animals can be fed with organic waste from various sources. Insects are highly efficient feed converters [1], produce less climate-damaging greenhouse gases than pigs and cattle [2], need less water and the risk of zoonosis is comparably small. Some insect species have a long history as human food but the high-quality insect proteins may also be extracted and used as a component in processed human food. Furthermore, insect proteins could play an important role in the replacement of soy and fish meal commonly used for animal feed. Also, lipids from insects may replace palm (kernel) oil and hence contribute to the conservation of tropical forests.

An insect with many positive properties is the black soldier fly, *Hermetia illucens* Linnaeus (Diptera: Stratiomyidae) (Figure 1). The slim fly measures up to 20 mm in length. The species is endemic in tropical and subtropical regions and is also present in southern Europe and parts of the Balkan Peninsula. It can be reared easily in mass cultures on almost all (decaying) organic matter. Even animal feces are ingested by the larvae and about 50% of this poor substrate is converted to a valuable larval biomass [3]. The black soldier fly prefers temperatures of about 28 °C for oviposition. The larvae hatch within 48 h and after about 2 weeks the larvae leave the slurry in search of a dry place to pupariate. After a further 2 weeks the imagines hatch. The imagines do not have mouthparts, therefore do not eat, sting or bite and are hence not vectors for diseases. The feeding activity of the black soldier fly is limited to the larval stage. The high number of eggs (500 to 1000), the short life cycle and the simple rearing conditions have made the larvae popular amongst aquarists and terrarium owners who use the larvae as excellent feed for reptiles or fish.

Sheppard has pioneered the work on *H. illucens* larvae and he recognized their value as feed for a variety of livestock more than 40 years ago [3, 4]. The quality of the dried larvae as feed rivals that of soybean meal. Other applications of the omnivorous *H. illucens* larvae have

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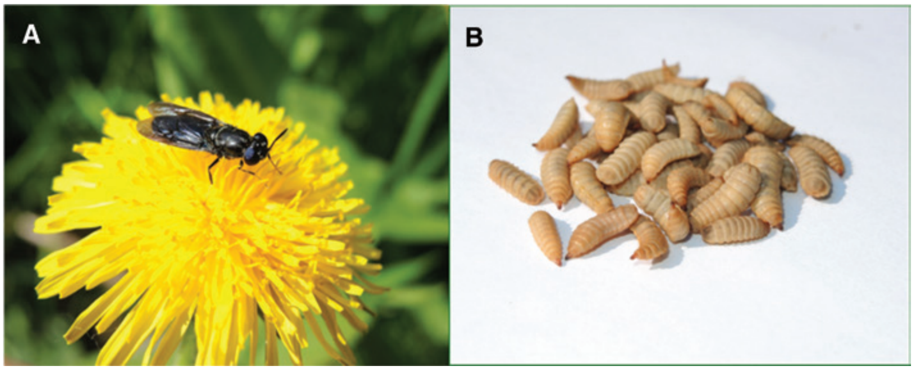

**Figure 1:** *Hermetia illucens*, the black soldier fly. (A) adult fly, (B) adult larvae shortly before pupation. [photographs: Hluschi & Gutzeit].

been reported. They may be used for manure reduction thereby lowering the nitrogen content [5]. As the black soldier fly colonizes manure in stables it helps in controlling the population of the house fly, *Musca domestica* L. [6–9]. It has been suggested that *H. illucens* larvae reduce pathogenic bacteria (*Escherichia coli* 0157:H7 and *Salmonella enterica*) in poultry manure [10].

On a small scale, home composting facilities have been developed (Biopod™) which make use of the waste-reducing activity of *H. illucens* larvae. The larvae have also been useful in forensic entomology to estimate post-mortem intervals [11–13]. Finally, studies investigating the use of *H. illucens* larvae for biodiesel production have been carried out [14].

In recent years many groups have tried to optimize and upscale *H. illucens* cultures to facilitate commercial applications. The production of protein and animal feed has been the focus of numerous companies that have been founded worldwide. Examples of companies that have already established *H. illucens* mass cultures include Hermetia Baruth GmbH (Germany), AgriProtein Technologies (South Africa) and Enterra Feed Corporation (Canada). According to Ilkka Taponen's entomology database there are 289 insect companies of which 222 are "active" [15]. Most companies that offer black soldier fly products are located in Europe (16) but there are also companies in Australia (1), Africa (4), Asia (6) and America (11) that offer these products. Unfortunately, EU law prohibits the use of animal products (including insects) as feed for livestock used for food production [16, 17] and this is a serious obstacle to economic success.

In September 2016, the European Commission Directorate-General for Health and Food Safety initiated discussions with EU member states with the aim of authorizing the use of insect proteins as feed for fish raised in aquaculture. To that end, the EU Executive prepared a series of proposals aimed at revising current provisions from the European Communities (Transmissible Spongiform Encephalopathies and Animal By-Products) Regulations. On 24 May 2017 the EU authorized the use of insect proteins in feed for aquaculture. The EU Commission formally adopted Commission Regulation (EU) 2017/893 [18] which is effective from 1 July 2017. This authorization is limited to a list of seven species including *H. illucens*, which must be fed with 'feed grade' substrates. Companies located outside the EU, such as Enterra Feed and AgriProtein, already offer insect products at local markets like "whole dried black soldier fly larvae", dried protein powder (i.e. MagMeal™), larval oil (i.e. MagOil™) and fertilizer (MagSoil™ and Soil+).

## 2 Sustainable production of proteins and lipids from mass cultures of *H. illucens* larvae

How can proteins and lipids from *H. illucens* be produced in a sustainable way without the use of agricultural land? We have established a small experimental production facility for *H. illucens* larvae using secondary resources exclusively. The principle of the sustainable production is illustrated in Figure 2. The energy required for the production of the larvae is provided by excess energy from a biogas facility.

It is our aim to establish a closed-circuit production unit in which only secondary resources are used and in which the input (energy and organic waste) are of little or no value for alternative applications. In our scheme, even the larval feces (larval liquid manure) can be recycled in the biogas facility and serve as fertilizer.

As the larvae generally live on substrate highly contaminated with microorganisms several studies have addressed the effect of the feeding activity by the black soldier fly larvae on bacteria and parasites in larval "feed

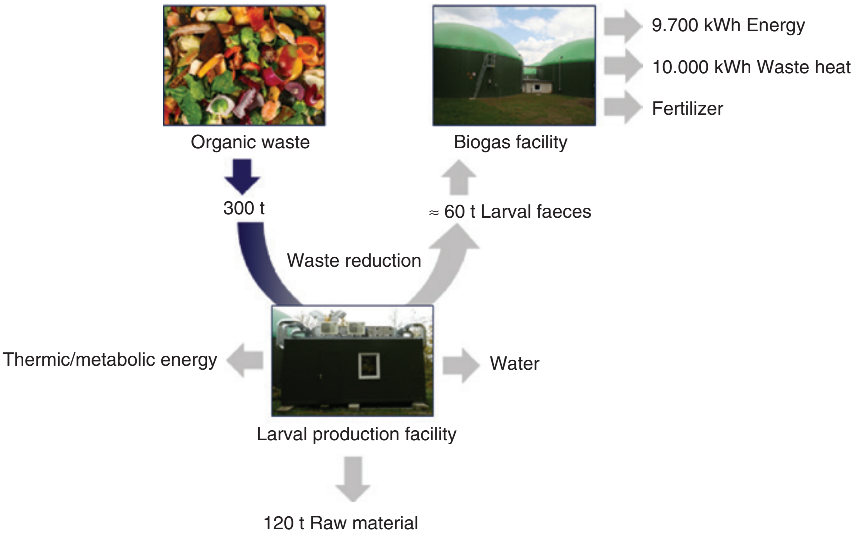

**Figure 2:** Pilot facility for sustainable production of *H. illucens* larvae from secondary resources. An example is shown for the production of 120 ton (t) larvae from 300 t of organic waste. Waste heat from a biogas facility is used as energy source for the mass culture. Larval feces can be recycled in the biogas facility or used directly as fertilizer. As a result, essentially no waste is generated in the production process.

and feces" [19]. When using the larvae as feed the microbiota should be controlled to avoid contamination with pathogenic bacteria.

In principle, the mass production of *H. illucens* larvae is technically simple. However, for large scale production the safety and efficiency of the culture is important and this requires zoological and technical expertise. The adult animals are kept in flight cages under optimal conditions regarding temperature, moisture and light. Eggs are collected from special deposition sites with suitable cavities to house the hundreds of fertilized eggs deposited by each fly. After about 2 days the larvae hatch. The larvae are placed on the feed in flat containers (to ensure sufficient oxygen supply). The temperature and moisture in the container must be controlled and the larval density is important. Ideally, the entire feed offered is ingested by the growing larvae. The metabolic activity is intense and hence much metabolic energy is produced. In the culture containers, the larvae tend to group together in the feed and, using thermography, we measured a local temperature of more than 45 °C (Figure 3). Due to the temperature differences, which cannot be impeded by mixing or shaking the culture, the larvae in the container will not develop in perfect synchrony.

After about 2 weeks the larvae reach the prepupal state and leave the feed which has turned into liquid manure by then. The larvae can be collected easily at that stage and if the design of the feed container provides a dry migration path out of the manure the larvae can be harvested essentially without any other contamination. For a large-scale production, other methods may be applied.

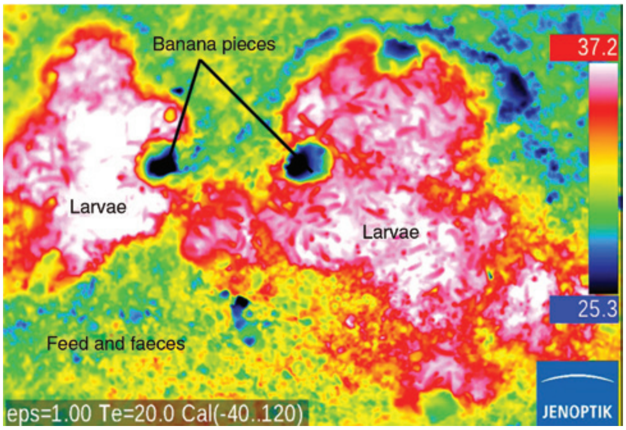

**Figure 3:** Thermographic documentation of the temperature distribution in larval mass cultures fed with banana pieces. The temperature within the groups of larvae can exceed 45 °C. (Photograph: Mueller 2016; VarioCAM® HD research 980 kindly provided by InfraTec GmbH.)

*Hermetia illucens* larvae could be used directly as chicken feed without any further processing if it were allowed by law. However, the now legalized use as fish feed in aquaculture requires processing as the high lipid content has to be reduced. When the larvae are processed and the valuable components are purified the respective products can be marketed separately. We have to keep in mind that the sustainable production of organic raw material cannot be cheaper than the ruthless exploitation of natural resources. For this reason, we paid attention to valuable bioactive substances in the larvae which offer additional benefits (Figure 4). Because of the enormous potential of antimicrobial peptides (AMPs) in insects for

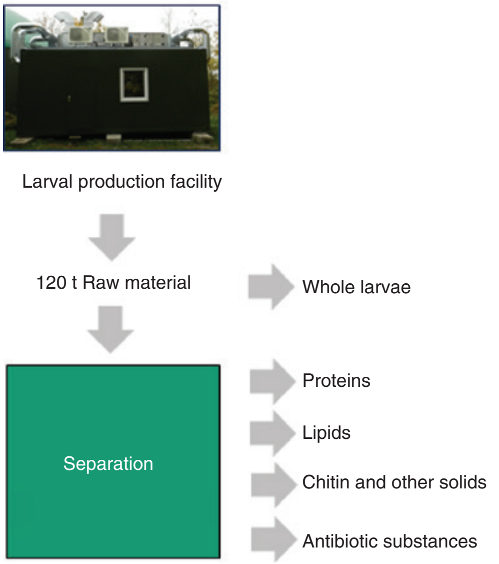

**Figure 4:** From 300 t of feed (Figure 2) about 120 t of larvae can be produced. The larvae can be used directly as animal feed. Alternatively, they can be processed and separated into valuable components like proteins, lipids, chitin and bioactive substances.

animal and human health, we describe the properties of these bioactive molecules in a separate section below.

The establishment of stable insect mass cultures is a challenge. There are, for example, several pathogens which may find optimal conditions for their propagation in *H. illucens* mass cultures. Examples include microsporidia, nematodes, apicomplexa parasites and miscellaneous other single-cell organisms which may be present in insect mass cultures, in particular when cultured under insufficient hygienic conditions. This problem has not yet been sufficiently covered in the literature [20]. The housefly *M. domestica* is a nutritional competitor in sparsely populated food containers, but more importantly, the flies may be vectors for pathogens. Furthermore, grain mites may act as feeding competitors while contamination with predatory mites (Figure 5) can severely damage the culture and usually requires the establishment of new mite-free cultures.

# 3 Characterization and quality assessment of proteins and lipids from *H. illucens* larvae

## 3.1 Proteins

**Figure 5:** Red mite on a black soldier fly larva breeding in a vermicomposting bin. Picture by Marilyn Sallee; picture number 5526412.

The protein content after dehydrating *H. illucens* larvae amounts to 42% in our cultures (unpublished data). The amino acid composition of proteins determines its quality as animal feed. The optimal amino acid composition may vary between different species due to different feed requirements and some amino acids may have to be supplemented. Otherwise, an amino acid imbalance may result in metabolic disorders [21]. According to Halver or Halver and Shanks, ten amino acids are essential for fish: arginine, histidine, isoleucine, leucine, lysine, methionine/cysteine, phenylalanine, threonine, tryptophan and valine [22, 23]. All of these amino acids are present in *H. illucens* larvae (Table 1, similar results to [25, 26]). The

**Table 1:** Amino acid composition (%) of *H. illucens* larvae compared to meal of herring and white fish [24] (red: essential; green: semi-essential amino acids).

| Amino acid | Black soldier fly [own results] | Herring meal | White fish meal |
|---|---|---|---|
| Alanine | 6.2 | 6.3 | 6.3 |
| Arginine | 6.2 | 5.8 | 6.4 |
| Aspartic acid | 10.3 | 9.1 | 8.5 |
| Cysteine | 0.5 | 1.0 | 0.9 |
| Glutamic acid | 12.2 | 12.8 | 12.8 |
| Glycine | 5.4 | 6.0 | 9.9 |
| Histidine | 4.8 | 2.4 | 2.0 |
| Isoleucine | 4.8 | 4.5 | 3.7 |
| Leucine | 7.7 | 7.5 | 6.5 |
| Lysine | 7.4 | 7.7 | 6.9 |
| Methionine | 0.6 | 2.9 | 2.6 |
| Phenylalanine | 6.2 | 3.9 | 3.3 |
| Proline | 6.2 | 4.2 | 5.3 |
| Serine | 4.1 | 3.8 | 4.8 |
| Threonine | 4.5 | 3.9 | 3.9 |
| Tryptophan | Not analyzed | 1.2 | 0.9 |
| Tyrosine | 6.0 | 3.1 | 2.6 |
| Valine | 6.7 | 5.4 | 4.5 |

amino acid analysis shows a typical amino acid pattern for animal proteins (Table 1) and *H. illucens* proteins may hence be regarded as a valuable source for animal feed. So far, we have no evidence that the amino acid composition can be altered significantly by different feeding regimes.

Compared with fish meal the content of essential amino acids (in % dry matter) in larval meal is considerably lower. However, the representation of essential amino acids in larval meal is very similar to fish meal [26]. This is of great interest to fish feed producers because an insufficient concentration of one essential amino acid leads to an impaired resorption of the other essential amino acids. In principle, *H. illucens* proteins can replace fishmeal in aquaculture and this is highly desirable for ecological reasons. Carnivorous fish require a feed with at least 45% protein content while for herbivorous fish 15%–30% protein content suffices [27]. Apart from the protein content the other feed components play an important role. For the rainbow trout, for example, the optimal protein and fat content should be 35% and 15%–20%, respectively [28]. As the *H. illucens* larvae are also rich in lipids (see below) this poses a problem and it is desirable to lower the fat content when used as commercial fish feed.

Due to their high protein content, *H. illucens* larvae could also be used as feed in poultry and pig breeding. Poultry, for example, needs 18%–20% crude protein with defined composition which cannot be reached by grain feed only [29]. Pigs need a crude protein content of 13%–21% depending on their age [30]. Generations of pigs and poultry have been raised with vegetarian feed (based on corn and soy bean meal) even though they are omnivorous animals and need animal protein. This leads to severe deficiencies because grains are typically low in lysine and legumes such as soybeans are low in methionine. With this combination of feed ingredients, methionine is typically the first limiting amino acid. One way of meeting the methionine requirement is to feed excessive protein so that the daily intake of methionine is met. This results in an undesirably high level of nitrogen excretion. The supplementation of synthetic methionine in poultry diets means less dietary protein is needed while the daily methionine requirement is still maintained [31].

### 3.2 Lipids

The lipid content after dehydrating *H. illucens* larvae amounts to 35% (unpublished data). The quality of lipids from *H. illucens* larvae is high and matches the quality of products from other animal and herbal sources, e.g. palm (kernel) oil (Figure 6). In contrast to the amino acid pattern the lipid composition can be altered by different larval diets. We showed that specific larval diets result in a lipid composition (with regard to carbon chain length) which resembles the composition of palm oil and palm kernel oil (Figure 6).

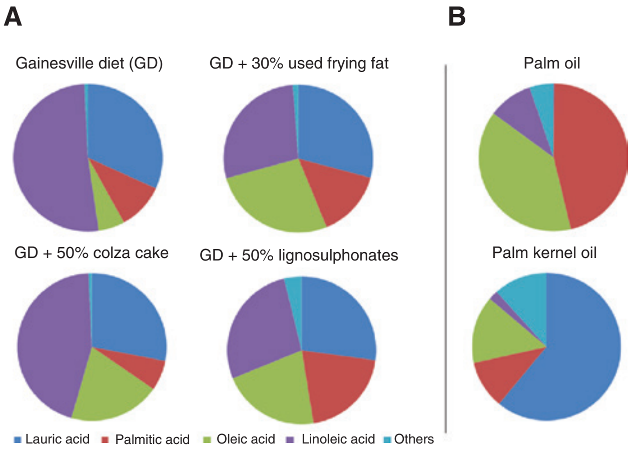

**Figure 6:** Feeding experiment regarding the influence of protein, fat and lignosulfonate intake on the fatty acid composition of *H. illucens* larvae in comparison to standard Gainesville diet (30% lucerne meal, 50% wheat bran, 20% corn meal which amounts to 15.3% protein, 3.8% fat, 12.6% fiber and 6.3% ash). (A) Effect of modified diets on fatty acid composition. (B) Data for reference oils (https://ndb.nal.usda.gov/ndb/).

As standard feed, we used the Gainesville diet [32]. By adding 30% frying fat to the larval diet, we were able to raise the palmitic and oleic acid fractions and to reduce the linoleic acid content significantly. Depending on the intended purpose (animal feed or industrial use) it is possible to "design" the desired larval fatty acid composition.

The high content of lauric acid (up to 50%), a raw material for many products in the cleaning and detergent industry, is remarkable. The content of the unhealthy erucic acid never exceeded 1.3%, regardless of the diet that was fed. Since 1977 the concentration for erucic acid in food is not allowed to exceed 5% in Germany [33].

## 4 Bioactive substances from *H. illucens* larvae and their potential use for animal and human welfare

### 4.1 Enzymes

Due to their omnivorous mode of life it stands to reason that *H. illucens* larvae contain various degrading enzymes of scientific and industrial interest. Up to now only the trypsin and chymotrypsin activity has been published [34, 35]. In 2014, Lee et al. furthermore heterologously expressed a novel cellulase gene from the gut microflora and characterized the resulting enzyme which shows 72% identity and 80% homology to the glycosyl hydrolase family 5 of *Dysgonomonas mossii* [36]. The presence of interesting enzymes like ligninases and cellulases in the larval gut depending on the feed composition is the subject of our current investigation. According to transcriptome analysis we have evidence that the larvae of the black soldier fly can produce cellulose- as well as chitin- and lignin-degrading enzymes (Heiko Vogel, MPI CE Jena, personal communication). We have carried out various in vitro enzyme activity tests regarding these enzyme activities but so far, we have not been able to show the respective activities in vitro. However, we were able to show trypsin and chymotrypsin activity in vitro also in homogenates of larval guts using photometry (Figure 7). The activity of these protein-degrading enzymes demonstrates that the biochemical processing of our larvae retains the enzyme activity.

## 4.2 Chitosan

When proteins and lipids are extracted from larval homogenates, an insoluble fraction remains including the insoluble cuticle. Its principle structural component is chitin, a polysaccharide consisting mainly of acetyl glucosamine sugar moieties. By alkaline deacetylation the water soluble oligomeric sugar chitosan can be prepared. The substance has interesting biochemical properties and many scientific and commercial applications for health care, agriculture, as well as cosmetic and textile industries have been reported. The substance is non-toxic, biologically degradable and antimicrobial and several biological activities have been reported which may be useful for the treatment of diseases including cancer, obesity and Alzheimer's disease. Chitosan also promotes tissue regeneration and has anti-inflammatory activity [37]. The effects on important cellular signalling kinases (MAPK and AMPK) suggest a complex interaction with cellular processes that are not yet fully understood. Chitosan efficiently binds lipids and this property is exploited in medical and technical applications. The biophysical properties of chitosan may also be used for the construction of vehicles for drug delivery and for many other industrial applications. Furthermore, chitosan O-, N-, S- and P-containing derivatives with desirable biological and biophysical properties can be prepared [38]. The worldwide production of chitosan comes largely from crustacean shells. However, the mass production of insects will provide an alternative source for this interesting substance.

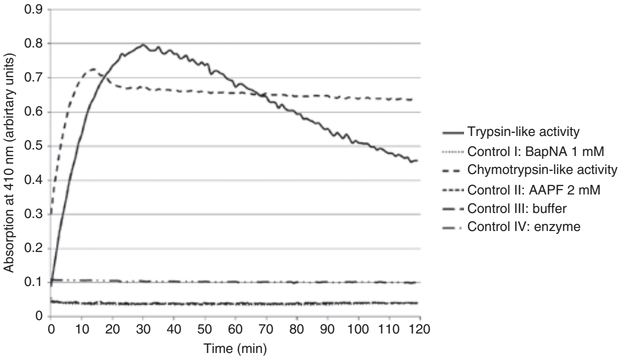

**Figure 7:** Enzyme activity of trypsin- and chymotrypsin-like enzymes in larval gut homogenates according to Kim et al. [34]. Substrates were N-Benzoyl-D, L-arginine-p-nitroanilide (BapNA, 83.3 μM) and Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (AAPF, 166.6 μM). Controls were enzyme assays with substrates without larval gut homogenates (I and II), assays with buffer (III) and assays with enzyme without substrates (IV).

## 4.3 Antimicrobial peptides from *H. illucens* larvae

The larvae of the black soldier fly, *H. illucens*, feed on a variety of decomposing organic substrates which are typically inhabited by a range of different microorganisms like bacteria and fungi. Survival in these habitats requires a powerful defense against microorganisms and hence it can be expected that the *H. illucens* larvae strongly express AMPs and other substances possessing antibiotic activity.

It is well known that insects have a well-developed innate immune system, subdivided into cellular and humoral defense responses. The latter involve the production of AMPs that are synthesized in the fat body and subsequently secreted into the hemolymph [39, 40]. In general, AMPs can serve as effective antibiotics or fungicides that mainly attack the cell envelope, especially the cell membrane and also affect intracellular targets of microorganisms which subsequently lead to cell death [41–43]. Several recent studies that reported the antimicrobial activity of the larvae hemolymph and maggot extract as well as of secretions, are promising for the development of new therapeutically valuable antibiotics, particularly in the defense of multi-resistant "super bugs" (e.g. the ESKAPE strains *Staphylococcus aureus* or *Pseudomonas aeruginosa*) [44–46]. In our lab, we used crude aqueous

*H. illucens* larval extract to study the bioactive effects on biofilms of several Gram-positive and Gram-negative microorganisms and to analyze the mode of action of *H. illucens* larvae AMPs on the Gram-positive model organism *Bacillus subtilis* in more detail.

### 4.3.1 Antimicrobial activity of *H. illucens* larval extracts on bacterial biofilms

A bacterial biofilm is a dynamic multicellular environment composed of sessile single-species or multi-species microorganisms attached to natural (biotic) or artificial (abiotic) surfaces [47]. Specific environmental conditions, e.g. nutritional limits or pH changes serve as a signal to develop biofilms [48]. Predominantly, mixed-species bacterial biofilms can be found in the environment whereas biofilms consisting of a single bacterial species mainly appear in connection with infectious diseases and other serious medical problems [48–50]. Despite the involvement of single-species biofilms in chronic infections when growing on abiotic surfaces, e.g. medical implants, catheters or contact lenses, there are also many hard-to-challenge infectious diseases caused by biofilms attached to the epithelium (e.g. chronic sinusitis or chronic osteomyelitis) [51, 52]. Additionally, microorganisms living in a biofilm are extremely resistant to antibiotic treatment reasoned by the bacteria-surrounding matrix that consists of extracellular polymeric substances (EPS) including proteins, polysaccharides and DNA [53, 54].

Biofilms of *P. aeruginosa*, a Gram-negative bacterium of the ESKAPE group with outstanding pathogenicity particularly for patients with cystic fibrosis or nosocomial infections, have been studied extensively [55–58]. *Pseudomonas aeruginosa* planktonic cells show resistance to nearly all available commercial antibiotics. Clearly, biofilms of that organism include large amounts of protecting EPS matrix that makes antibiotic treatment of *P. pseudomonas* ineffective. The same holds true for the Gram-positive pathogen *S. aureus*. In this case it has been reported that *S. aureus* and relatives (e.g. *Staphylococcus epidermidis*) cause 60% of the nosocomial infections and show the highest resistance to currently used antibiotics when growing as biofilms [50, 59]. Therefore, it is of the utmost importance to find new potent antibiotics to challenge these "super bugs". Very promising drugs to combat multi-resistant strains are represented by antimicrobial peptides derived from eukaryotes made by the innate immune system of the producing organism. In recent years several AMPs were discovered for their ability to affect bacterial biofilms [60]. For example, the cathelicidin LL-37 produced by human neutrophils is very effective against *P. aeruginosa* biofilm formation and already existing biofilms of the multidrug-resistant *S. aureus* [61–64]. Despite these advantages, currently no commercial antibiofilm peptides are available to challenge biofilm infections specifically [60].

To investigate the antimicrobial effects of *H. illucens* larval extracts on microbial biofilms, we prepared aqueous larval extracts according to the protocol from Park et al. [65]. The protocol from O'Toole was used to test the antimicrobial activity of aqueous larval extracts on *E. coli*, *Micrococcus luteus*, *Pseudomonas fluorescens* and *B. subtilis* biofilms grown in microtiter plates, respectively [66]. The antibiotic chloramphenicol and the chemical compound hypochlorous acid served as positive controls. After a defined incubation period, crystal violet was added and the remaining biofilms were quantified by measurement of absorbance at 550 nm. We found a strong biofilm degradation (reduction of 70%–90%) for all tested microorganisms (see Figure 8A) which is likely due to the

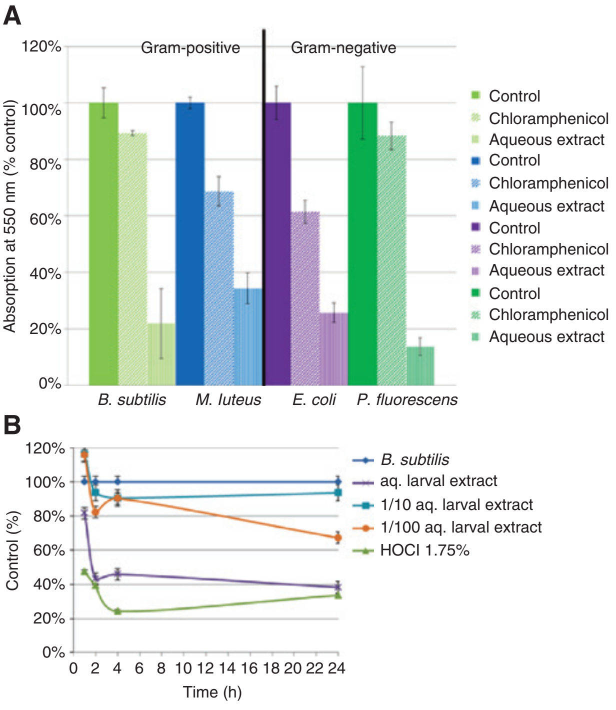

**Figure 8:** Effect of aqueous *H. illucens* larval extracts on microbial biofilms. (A) Antimicrobial effect of aqueous *H. illucens* larval extracts (28 mg larvae/well) and chloramphenicol (40 µg/well) to bacterial biofilms. Biofilm reduction was visualized by absorbance measurement at 550 nm. (B) Time-resolved degradation of *B. subtilis* biofilms treated with several dilutions of aqueous *H. illucens* larval extract (130 mg larval equivalent) and 1.75% hypochlorous acid (control) (n = 9).

antimicrobial compounds available in the aqueous *H. illucens* larval extracts. Furthermore, the biofilms of the Gram-positive bacteria *B. subtilis* or *M. luteus* are affected in the same manner as the biofilms of the Gram-negative bacteria *E. coli* or *P. fluorescens*. This is remarkable because so far only an antimicrobial effect of the *H. illucens* defensin DLP4 on Gram-positive microorganisms has been reported [67]. DLP4 is clearly not the only AMP in the crude larval extracts which we used in our experiments. The strong effects on *B. subtilis* biofilms even when compared to 1.75% hypochlorous acid (Figure 8B) indicate that several AMPs are likely to act in concert.

In future experiments isolated *H. illucens* AMPs and mixtures of defined antibacterial substances will have to be tested for their activity against biofilms. The experiments carried out so far provide evidence for the great potential of this approach.

### 4.3.2 Classes of AMPs in *H. illucens* larvae

Little is known about the AMPs of the black soldier fly. Very recently, Elhag et al. published the identification of seven genes encoding for AMPS in the genome of *H. illucens*. Additionally, the authors also described the successful expression and functional analysis of stomoxynZH1 from *H. illucens* which exposed antimicrobial activity against *E. coli* and *S. aureus* [68]. However, the available data from different insects indicate that the families of AMPs are largely conserved between insects and only the number of AMPs may differ between the species depending on their respective habitats. The available information about insect AMPs sheds some light on the biological activities to be expected in *H. illucens* larvae and hence the most important findings are summarized in the following sections.

The antibacterial activity of AMPs from dipterans was first observed in the hemolymph of the fruit fly *Drosophila melanogaster* by Robertson and Postlethwait who also pointed out that insects previously infected with microorganisms develop a stronger humoral immune response accompanied by an increased synthesis of AMPs to counteract microbial infections [69]. Microarray studies of bacteria-preimmunized crude *H. illucens* larval extract revealed a high expression of several AMPs belonging to the classes defensins (4.3.2.1), cecropins (4.3.2.2), attacins (4.3.2.3) and diptericins (4.3.2.4) (Ariane Müller, unpublished results). Typically, these classes of AMPs can be found in dipteran insects and have in common that they are cationic or basic but differ in structural and sequence properties [70].

#### 4.3.2.1 Defensins

The insect defensins, a family of small (~4 kDa) cysteine-rich cationic peptides, contain six conserved cysteines that form three intramolecular disulfide bonds. The insect defensin family also shows a strong homology to defensins of mammals. The typical structure of insect defensins consists of an N-terminal loop, an α-helix and two or three antiparallel β-sheets [70]. The majority of insect defensins have strong antimicrobial activities against Gram-positive microorganisms only, such as *B. subtilis* or *S. aureus*. In some rare cases, insect defensins can also kill Gram-negative bacteria and/or fungi. Defensin and drosomycin from the dipteran insects *Anopheles gambiae* and *D. melanogaster*, respectively, show very effective antibacterial (Gram-negative) and antifungal activity [71, 72]. Defensins from insects target the cytoplasmic membrane of microorganisms by interaction with phospholipids, followed by forming channels that result in membrane permeabilization and subsequently death of the microbial cell [70, 73, 74].

#### 4.3.2.2 Cecropins

The small cationic cecropin peptides exhibit a broad antimicrobial activity against Gram-positive and Gram-negative microorganisms as well as against fungi [70]. Cecropin from the lepidoptera *Hyalophora cecropia* was also the first antimicrobial peptide that was purified successfully [75]. Remarkably, the cecropin-like peptide stomoxyn from the dipteran blood-sucking fly *Stomoxys calcitrans* also shows antiparasitic activity against trypanosomes [76]. It has been known that the antimicrobial activity of cecropins is also reasoned by the amidation of the C-terminus of the peptide which is important for the interaction with liposomes of the attacked microorganism [77, 78]. Cecropins are bactericidal active as helical structures in a hydrophobic environment (e.g. the bacterial cell membrane or lipopolysaccharides). Typically, cecropins consist of an N-terminal amphipathic α-helix that is linked to the C-terminal hydrophobic α-helix by an intramolecular hinge region [79]. Both the amphipathic and the hydrophobic α-helix contain amino acid residues that are essential for interaction with bacterial membranes and lipopolysaccharides [70, 80].

#### 4.3.2.3 Attacins

Attacins are very large (~20 kDa) glycine-rich helical AMPs and can be found in two isoforms, basic and acidic attacins depending on their isoelectric points [70]. Structurally, matured attacin peptides consist of an N-terminal attacin domain and two glycine-rich domains [70, 81]. Predominantly, attacins are antimicrobial active against

*E. coli* and some other Gram-negative bacteria, e.g. *Acinetobacter calcoaceticus* and *Pseudomonas maltophilia* [82]. Antibacterial activity against Gram-positive bacteria, fungi or parasites has been detected in a few cases only. For example, attacin-A1 from the dipteran tsetse fly *Glossina morsitans* also kills the parasite *Trypanosoma brucei* [83]. The mechanism of antibacterial action (MOA) of attacins is characterized by the binding to lipopolysaccharides and inhibiting the synthesis of outer membrane proteins of Gram-negative bacteria which leads to a higher permeability and instability of the microbial cell envelope [70].

#### 4.3.2.4 Diptericin

The 82 amino acid long peptide diptericin was originally isolated from the dipteran northern blow-fly *Phormia terraenovae* and showed no direct homologies to the known AMPs from insects (e.g. cecropins or attacins) [84, 85]. Thus, diptericins are grouped as large *O*-glycosylated peptides lacking an ordered secondary-tertiary structure whereas the glycosylation is not compulsorily required for antimicrobial activity but rather to prevent the degradation of the peptide in the insect's hemolymph. However, diptericins inhibit the growth of the Gram-negative bacteria *E. coli* and *Salmonella typhimurium* in a very short time (~15 min) without lysing mammalian blood cells (e.g. sheep erythrocytes) [86]. It has been assumed that diptericins form their specific structure in complex with the target molecule only but the exact MOA of diptericins remains elusive [86].

Despite the lack of a defined MOA for diptericins, the other insect AMPs described above likewise attack the cell envelope particularly the cell membrane of bacteria. The Gram-positive microorganism *B. subtilis* is a well-established model organism which has been graded with the generally recognized as safe (GRAS) status and has "easy to handle" and efficient molecular and genetic properties. Furthermore, cell wall biosynthesis and signaling pathways essential for the integrity of the cell envelope and survival of *B. subtilis* have been extensively investigated [87–93]. Therefore, *B. subtilis* is highly suitable to study the mode of action of insect AMPs on the bacterial cell membrane in more detail.

### 4.3.3 Antimicrobial peptides of *H. illucens* larvae induce the cell envelope stress response of *B. subtilis*

The bacterial cell envelope represents the first barrier against damaging environmental conditions. In its natural habitat, the soil, *B. subtilis* lives in permanent competition with other microorganisms for the best living conditions. As a defense mechanism, many microorganisms including *B. subtilis* produce AMPs to counteract competing microorganisms. Many of these antibiotic substances mainly attack the bacterial cell envelope. Thus, a constant monitoring of the cell envelope integrity is crucial for survival of the bacterial cell. In *B. subtilis*, a network called the cell envelope stress response (CESR) is responsible for detecting signals affecting the integrity of the cell envelope and to transduce the signals into an appropriate cellular response leading to cell envelope protection by establishing repair mechanisms and antimicrobial resistance. The ABC transporter associated two-component systems as resistance determinants play an important part in the CESR in *B. subtilis* and have been well investigated [94–96]. In our lab, we use these signal transduction pathways for the development of whole-cell *B. subtilis* biosensors that specifically respond to different classes of AMPs. Additionally, these biosensors are highly feasible to characterize newly found antibiotics regarding their MOA [97].

Recently, we found that the ABC transporter associated ApeRS two-component system (formerly known as YxdJK) is highly induced after treatment of *B. subtilis* cells with aqueous *H. illucens* larval extract (Diana Wolf, unpublished data). In the first experiment, we used aqueous extracts of non-immunized and preimmunized (with *B. subtilis*) *H. illucens* larvae to treat *B. subtilis* cells containing the Ape-biosensor that include the $P_{ape}$ target promoter fused to luminescence genes. The biosensor signal transduction in response to a signal occurs via autophosphorylation of the membrane-anchored histidine kinase ApeS and phosphate transfer to its cognate response regulator ApeR (Figure 9A). Subsequently, the activated response regulator binds to its target promoter $P_{ape}$ which drives the expression of a reporter gene (luminescence) (Figure 9A). So far, it has been known only that the Ape-system responds to the human cathelicidin LL-37 [98]. A great variety of AMPs of prokaryotic origin we tested, did not induce the Ape-system. To test the induction of the Ape-biosensor by *H. illucens* larval extract, *B. subtilis* cells were spread onto agar plates. Subsequently, Whatman paper disks soaked with certain aqueous larval extracts were also placed onto the agar plate. The plate was incubated overnight and afterwards analyzed for luminescence due to $P_{ape}$ promoter activity by detection in a luminescence machine ("Moon shine assay"). We found a strong activity of $P_{ape}$ visualized by a strong luminescence signal when *H. illucens* extract of preimmunized larvae was used (Figure 9B). The bioluminescence signal of $P_{ape}$ was much weaker in response to non-immunized *H. illucens* larval extract reasoned by the

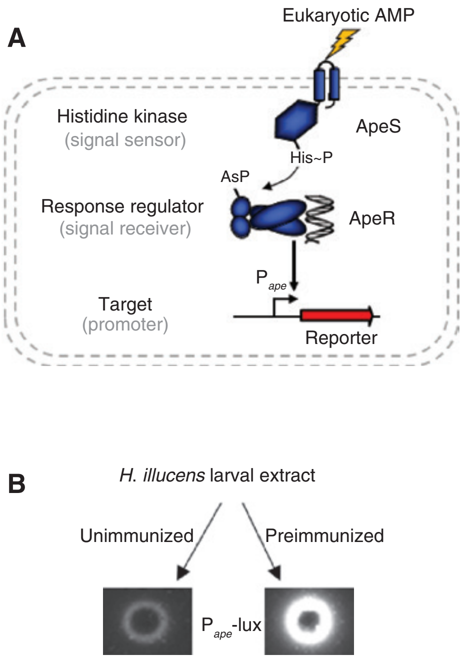

**Figure 9:** The Ape-biosensor of *B. subtilis*. (A) Genetic organization of the whole-cell biosensor ([97] adapted). (B) Induction of the target promoter $P_{ape}$ after treatment with *H. illucens* larval extract.

absence of a specific larval immune response to a certain antigen (e.g. *B. subtilis*) (Figure 9B).

In summary, we assume that the Ape-biosensor exclusively detects and responds to eukaryotic AMPs but the exact compound specificity of the Ape-biosensor remains elusive. Furthermore, we expect that the Ape-system, as part of the CESR, plays an important cellular role as resistance determinant in bacterial membrane protection because LL-37 as well as insect's AMP mainly affect and disrupt the bacterial cell membrane. As described in the previous section, insect's defensins and cecropins are very efficient at counteracting Gram-positive bacteria (e.g. *B. subtilis*). Therefore, further experiments regarding the purification of the crude larval extract by chromatography as well as to test pure AMPs from other dipterans together with the Ape-biosensor are currently in progress.

## 5 Summary and outlook

In recent years insect biotechnology has been extraordinarily successful. This is reflected not only in the intensity of research but also in the commercial potential that has become apparent from the increasing number of newly founded companies in this field. The commercial potential using insect products is based on the evolutionary success of insects. The black soldier fly, *H. illucens*, illustrates this nicely. The insect is robust and survives under harsh conditions and is remarkably resistant to bacterial infections. This makes mass cultures attractive as it is possible to raise the larvae with low quality feed and yet the feed conversion ratio is high. The production of proteins and lipids from insects is ecologically desirable as it can be carried out using only secondary resources and hence contributes to the conservation of valuable ecosystems. Legal restrictions in the EU concerning the use of animal proteins in feed limit the large-scale production of insect raw material at present.

The bioactive substances in *H. illucens* larval extracts are of great interest and will play an important role in the future in making insect mass cultures economically successful. We have paid particular attention to unusual enzymes in *H. illucens* larvae which might be useful in the processing of cellulose, lignin and other inert organic material. Furthermore, the potent antibacterial peptides in the black soldier fly and in other insects hold great promise in view of the highly problematic emergence of bacterial strains resistant to common antibiotics that are used to fight human and animal infections.

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