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MINIREVIEW

Microbial Community Dynamics during Rearing of Black Soldier Fly Larvae (*Hermetia illucens*) and Impact on Exploitation Potential

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ABSTRACT The need to increase sustainability in agriculture, to ensure food security for the future generations, is leading to the emergence of industrial rearing facilities for insects. One promising species being industrially reared as an alternative protein source for animal feed and as a raw material for the chemical industry is the black soldier fly (*Hermetia illucens*). However, scientific knowledge toward the optimization of the productivity for this insect is scarce. One knowledge gap concerns the impact of the microbial community associated with *H. illucens* on the performance and health of this insect. In this review, the first steps in the characterization of the microbiota in *H. illucens* and the analysis of substrate-dependent dynamics in its composition are summarized and discussed. Furthermore, this review zooms in on the interactions between microorganisms and the insect during *H. illucens* development. Finally, attention is paid to how the microbiome research can lead to alternative valorization strategies for *H. illucens*, such as (i) the manipulation of the microbiota to optimize insect biomass production and (ii) the exploitation of the *H. illucens*-microbiota interplay for the discovery of new enzymes and novel antimicrobial strategies based on *H. illucens* immunity using either the whole organism or its molecules. The next decade promises to be extremely interesting for this research field and will see an emergence of the microbiological optimization of *H. illucens* as a sustainable insect for industrial rearing and the exploitation of its microbiome for novel biotechnological applications.

KEYWORDS *Hermetia illucens*, biotechnology, industrial microbiology, insect-microbial interactions, microbiota, natural antimicrobial products

The expanding world population is a major challenge for our food system, which struggles to ensure global food security in a sustainable manner. One issue is the high numbers of inefficiencies and losses during agricultural production (1). In 2011, the FAO estimated that worldwide, almost 1.3 billion tons of food per year is lost or wasted (2). A recent study estimates that only 24.8% of the harvested biomass is used for human food consumption and defines livestock production as an important factor in these losses (1). Considering that the global demand for protein is expected to rise more than 75% over the current demand by 2050 (3), more efficient animal production and alternative protein sources are required to avoid a protein gap. Another issue is our consumer behavior of food overconsumption and excessive food wastage (4). Over time, the loss of nutrients at the production and consumer levels will disturb the sustainable regeneration capacity of our planet (5). Hence, innovative strategies are needed that transition our current food system toward a circular sustainable system

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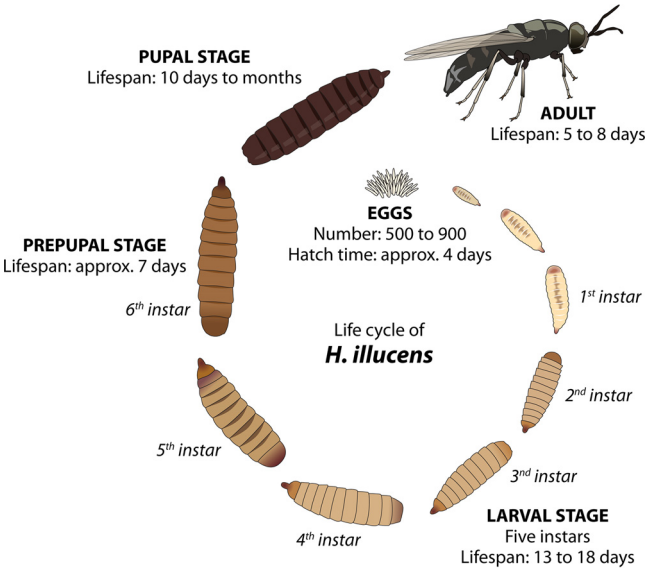

FIG 1 Life cycle of *H. illucens*. The different stages of the development of *H. illucens* are shown, as well as the average duration for each of these different stages.

that also focuses on reintroducing nutrients in the system by the conversion of waste to biomass.

The industrial rearing of specific edible insect species is a promising strategy, as insects offer (i) an energy-efficient high-quality protein source for feed/food (6, 7) and/or (ii) a sustainable strategy to upcycle various waste streams by converting them into biomass (8). The black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomyidae), has become one of the most important insects in the world for bioconversion (9), being reared by multiple companies on an industrial scale of metric tons per week or month. *H. illucens* is characterized by a short life cycle of 40 to 45 days (10). In brief, after approximately 4 days, a larva emerges from an egg and undergoes five instars as a larva in 13 to 18 days, followed by prepupal and pupal stages from which the adult fly emerges. The adults no longer feed and live for about five to 8 days during which they mate. The female then lays 500 to 900 eggs (Fig. 1). The larvae of this insect are able to process a wide range of organic substrates and turn them into body mass mainly composed of protein, fat, and chitin (8). The high-quality protein can be used as feed, while the other compounds can be used as a raw material in the chemical industry (11, 12). The fat fraction can be extracted and converted into biofuel (13), while the chitin can be processed to chitosan that is used in a range of applications from wastewater treatment to bioactive coatings (14). Currently, specific legislative rules concerning the use of this insect are largely missing across the world. However, in several countries, this situation is changing (15). Beginning in July 2017, *H. illucens* larvae are authorized by the European Commission as a feed ingredient in aquaculture (16), and by 2020, insect meal is expected to be approved for pig and poultry feed as well (17). In the United States, whole dried *H. illucens* larvae are allowed as feed for salmonids by the FDA (AAFCO report, 19 January 2016) and three states (Idaho, Indiana, and Alaska) go even further, allowing the larvae as feed for all species. Furthermore, the FDA approval for the use of dried larvae in chicken broiler/layers feed is also pending for 2018. In response to the potential for this insect and the opening of new markets, several *H. illucens* breeding companies in the world are scaling up and optimizing production. These developments led to the establishment of *H. illucens* as an insect for industrial rearing, and it was also given the status of a farm animal in the legislative framework in Europe (EG no. 1069/2009) (6).

While extensive scientific research has already led to an optimization of the productivity and feed efficiency for most traditional farm animals (e.g., poultry, pigs, and

ruminants), the impact of various parameters on production for insects, including *H. illucens*, is mostly uncharted terrain. For example, it is well established in traditional farm animals that the microbiota in the gastrointestinal tract (GIT) contributes significantly to animal performance and health and that its composition is highly variable depending on the feed (18). Such knowledge was lacking for industrially reared insects, and this sparked the emergence of a novel field of research, which is exemplified by the steady increase in scientific output regarding this organism. Unfortunately, most studies focus on the impacts of the environment and diet on *H. illucens* development and its nutritional value (19), often neglecting the roles of the microbiota of these substrates and of the insect itself in the growth performance. Nevertheless, the ability of *H. illucens* larvae to grow on a range of organic substrates (8, 20), in different stages of decay, raises intriguing questions toward the role played by the microbiome in this ability and the mechanisms in place that prevent the microbial community from being taken over by noncommensal bacteria in the insect gut. To this end, we summarize and discuss the available reports that characterize the microbial communities in *H. illucens* and/or investigate their dynamics on different substrates to assess the interplay between microorganisms and the insect. The initial observations in this emerging field not only illustrate the important role that microorganisms play during *H. illucens* development but also shed light on the immune mechanisms in *H. illucens* that enable this organism to grow even in highly microbe-contaminated substrates. As such, the current state of the art offers a perspective toward the potential in exploiting and manipulating the microbiome of *H. illucens* toward better zootechnical performance and insect-derived antimicrobial strategies. This review aims to inspire researchers and incite future research toward the microbiological optimization of *H. illucens* rearing, its sustainable nature, and the exploitation of its microbiome for the discovery of novel biotechnological applications.

GUT MICROBIOME

In domestic animals, the gastrointestinal tract (GIT) harbors dense and complex microbial communities, composed of bacteria, protozoa, fungi, archaea, and viruses. These communities were found to be involved in (i) the digestion and fermentation of plant polymers, (ii) the synthesis of vitamins, (iii) the bioconversion of toxic compounds, (iv) the stimulation of the immune system, (v) the maintenance of gut peristalsis, (vi) the maintenance of intestinal mucosal integrity, and (vii) the formation of a barrier against colonization by pathogens (21). Research also revealed that its composition is affected by numerous factors, such as feeding practices, diet, and farm management. Alterations in the microbiota can affect feed efficiency and the welfare and health of the animals (18). In comparison, the GITs of most insects also harbor high numbers of bacteria, outnumbering insect cells by 10-fold (22). Furthermore, these bacteria also play several functional roles, such as enabling the digestion of specific compounds and distorting the sex ratio in some insects. It may be postulated that the general principals concerning microbial gut health apply in insects as well (23).

The GIT of *H. illucens* can be divided into three parts, namely, the foregut, the midgut, and the hindgut, and all three can host different bacterial communities. While it can be assumed that microorganisms fulfill similar functions in the GIT of this organism as in other farm animals, experimental data are scarce (24, 25). It is an issue that undoubtedly will be addressed during the further implementation of this insect in industrial rearing. From a broader perspective, the fact that *H. illucens* larvae can digest a wide range of organic substrates, even during decay, raises questions toward the role played by the GIT microbiome in this versatility and the mechanisms in place that prevent the microbial community from being taken over by unwanted bacteria.

IMPACT OF SUBSTRATE COMPOSITION ON *HERMETIA ILLUCENS* DEVELOPMENT

The diversity of substrates reported in the literature on which *H. illucens* larvae can grow is remarkable and ranges from agricultural by-products, such as potato steam peelings, bread remains, or sugar beet pulp, seaweed, liver, and cadavers to animal and

human manure (12, 26–30). However, while the larvae can grow on this wide range, the substrate impacts not only the time needed for *H. illucens* to progress through its life stages but the nutritional composition of the larvae as well (19, 27, 29). For example, larvae grown on a protein- or fiber-rich substrate were shown to develop much slower than larvae grown on a balanced diet of cereal processing leftovers (27). Their mass yield was only 36 or 17%, respectively, of the mass yield achieved by the larvae on the control diet. The larvae on the fiber-rich diet even ceased their development (27). Aside from affecting larval mass gain, another study showed that altering the ratio between cow manure and fish offal in the substrate results in an altered fatty acid composition of the larvae (31). Other specific compounds present in the substrate, be they lipid- or water-soluble, can also accumulate in the larvae, as was shown for vitamin E and iodine in larvae fed seaweed (28). These examples prove that it is feasible to tailor the nutritional composition of the larvae to some extent and at the same time illustrate the possible accumulation of unwanted compounds. However, studies have shown that this is mainly an issue for specific metals, while none of the toxins and pesticides tested so far have been shown to accumulate (32–35).

As an explanation for the substrate-dependent growth variations, researchers often solely refer to the different constellations of macronutrients between these substrates. However, it is crucial to investigate the roles of other variables that differ between these substrates as well. One example is the moisture level of the substrate. Recently, it was shown that low moisture contents in feed diminish larval growth (36, 37). Larvae appear to be unable to develop with a moisture content of $<$40% (37). Another key variable between substrates is the compositions of their microbial communities. Yet, none of the studies mentioned above take into account the microbiota of the substrate. One study that reports a superior growth and survival rate for *H. illucens* larvae isolated from the wild compared to that of larvae reared on three well-balanced substrates does hint at a role for anaerobic bacteria (10). They were suggested to be competitors of the larvae for nutrients in the substrate. During rearing, the substrate is typically not removed, enabling anaerobic bacteria to develop (10). The authors attribute the superior growth of the wild-harvested larvae to their capability to scavenge fresh nutrient sources during their growth. However, the exact nutritional value of the available manure or of the defined diets was not determined, leaving the question of whether fresh manure indeed has more nutritional value then the aging defined diet. Alternatively, we postulate that the better performance of the wild larvae is due to their ability to feed on substrates with a more complex or larger microbiota. These substrates might be more suited to stimulate larval growth, while the larvae on the defined diets are faced with increasing competition from unwanted bacteria in the aging substrate. Likewise, communications with several industrial BSF larvae rearers have pointed out that sterilized substrates do not enable larval growth but need to be inoculated with a small amount of untreated substrate from a previous rearing cycle in order to obtain proper zootechnical performance (unpublished data).

These observations illustrate that microbiota do seem to play a crucial role during *H. illucens* growth. To explore this role of the microbiota, it is of the utmost importance to characterize the microbiota of the substrates and the larvae during rearing in quantitative and qualitative ways and to investigate their interactions and the consequent impact on larval development.

MICROBIAL COMMUNITY OF *HERMETIA ILLUCENS* LARVAE ON NATURAL SUBSTRATES

The first report of the analysis of a bacterial community in *H. illucens* larvae was published in 2011 and aimed to characterize the gut microbial community during laboratory rearing on three different substrates (food waste, calf forage, and cooked rice). The goal was to investigate the role of the intestinal microorganisms in the remarkable catabolic abilities of these larvae (38). To this end, larvae underwent three washing steps with 70% ethanol to enable the sterile removal of the entire gut. Next, the 16S rRNA gene sequences of the gut microbial metagenome were analyzed using

TABLE 1 Composition of microbial communities of *H. illucens* larvae on various substrates

Study authors and year of publication	Life stage	Substrate[a]	Bacterial community structure	
			Major phylum	%
Jeon et al., 2011 (38)	8-day-old larvae	Food waste	*Bacteroidetes*	67.36
			Proteobacteria	18.85
			Firmicutes	9.4
			Fusobacteria	2.01
			Actinobacteria	1.92
		Cooked rice	*Proteobacteria*	54.01
			Firmicutes	42.27
			Unclassified	3.52
		Calf forage	*Proteobacteria*	31.08
			Actinobacteria	24.58
			Firmicutes	23.46
			Bacteriodetes	20.51
Zheng et al., 2013 (39)	7-day-old larvae	Gainesville diet	*Bacteroidetes*	54.40
			Firmicutes	20[b]
			Proteobacteria	16[b]
			Actinobacteria	9[b]

[a]Substrates that have been tested so far: food waste from Korean restaurants, cooked rice, calf forage (crude protein, 15%; crude fat, 15%; crude ash, 1.5%; Ca, 0.8%; P, 0.8%; and total digestible nutrients, 67%), and Gainesville diet (20% corn meal, 30% alfalfa meal, and 50% wheat bran, saturated with water).
[b]Estimated on the basis of a visual representation in the cited article.

pyrosequencing, and substrate-dependent shifts in the community structure of the GIT bacteria were found. Furthermore, the identified communities were quite unique compared to those of the intestinal microflora from other insects, e.g., termites. The overall diversity in the community was found to be linked to the nutritional complexity of the substrate, with more diverse nutrients being present in the food waste than in the other substrates. This led to the identification of 176 unique species in the food-waste-fed larvae compared to 41 and 36 species in the calf-forage- and cooked-rice-fed larvae, respectively. Interestingly, a total of 36 bacterial species were identified in all three substrates. This points toward the existence of a unique core composition in the gut microbiota, although the authors stated that more research is crucial. Analyses using 16S rRNA gene pyrosequencing of the bacterial diversity during different life stages of *H. illucens* add weight to the existence of such a core microbiota (39). Of the 78 bacterial genera found in this study, 20.5% were shared by the larval, prepupal, and pupal stages. Of the total, 11.5% even persisted into adulthood, clearly illustrating the capacity of specific bacteria to be retained through successive life stages (39). Of all the genera present on the eggs, 5% were also found on the emerging adults. One species, of the genus *Providencia*, is highly likely transmitted vertically, as it represented 49% of the identified *Gammaproteobacteria* hits across all life stages. It might achieve this by residing in the hemolymph of the larvae, as has been shown for other *Providencia* spp. in *Drosophila melanogaster*. As this study performed metagenetics on entire specimens without any surface sterilization, it is also possible that these bacteria reside on the surfaces of the deposited eggs. This hypothesis has not been tested further to date, to our knowledge.

Table 1 lists the compositions of the microbial communities of 7- and 8-day-old larvae at the phylum level and shows the clear differences in microbiota between the various substrates. However, what is also interesting is that approximately 80% of the compositions of the microbiota are altered over the subsequent larval stages in the second study, even on an identical diet, illustrating that the compositions of these bacterial communities are not a mere reflection of the communities present in the substrate and that additional mechanisms play a role (39). For example, in the burying beetle (*Nicrophorus vespilloides*), major portions of the midgut are devoid of any bacteria, revealing the existence of sanitization mechanisms in the insect gut (40). It will be interesting to explore if such spatial differences also exist in the *H. illucens* larval gut

to exploit the potential to preselect the microbiome toward the digestion of specific substrates.

The fungal communities in the GITs of *H. illucens* larvae were also recently profiled using 454 pyrosequencing on the amplicons of the 5.8S-internal transcribed spacer (ITS) rRNA region (41). Larvae were reared in the laboratory on chicken feed solely for 17 or 21 days or for 17 or 14 days followed by 4 or 7 days, respectively, on vegetable waste. Then, the entire guts from eight surface-sterilized larvae per condition were dissected. The identified population compositions illustrate that the fungal community is impacted by substrate alterations in a similar manner as the bacterial community. The fungal diversity increases upon feeding with a substrate composed of various waste streams. However, fungal communities seem to be more likely than bacterial communities to be overtaken by a single genus depending on the feeding conditions. In this case, *Pichia* (93.7%), *Geotrichum* (90.3%), or *Trichosporon* (87.7%) dominated the communities in three of the five feeding regimes tested. This can be due to the fact that many yeasts are able to inhibit the growth of others by using antagonistic mechanisms, such as antimicrobial compounds (42).

These metagenetics studies offer some initial insights on the structures and dynamics of microbial communities and, at the same time, illustrate how little is known about the compositions and the substrate-dependent dynamics of these communities. To shed light on these dynamics, more data sets are needed and these data sets, especially when obtained with metagenetics, must be interpretable with a high confidence level. In this regard, the improved read length of Illumina sequencing and the use of zero-radius operational taxonomic units (zOTUs), which are believed to better represent the true biological variation in a sample (43), are two evolutions that in the future will improve the often limited confidence for identification at the species level. Finally, the reported studies were all performed on larvae grown under laboratory conditions. It would be interesting to find out if and how industrial rearing on the same or other substrates impacts the microbiota. As industrial rearing often implies less controlled and/or less hygienic environments, it can be questioned whether and how the microbial loads of the substrates and larvae evolve over time, in the same way as recently being studied for the lesser mealworm, *Alphitobius diaperinus* (44). The study revealed higher microbial loads in the culture medium (containing feed, feces, and skin sheds) than in the larvae and uncovered the dominance of specific bacterial species over time in the guts of the mealworms. In the future, sampling from several industrial producers and at various time points during the rearing process could offer insights into these research questions for *H. illucens* as well.

FUNCTIONS OF THE MICROBIAL COMMUNITY DURING THE LIFE CYCLE OF *HERMETIA ILLUCENS*

What the exact functions of these bacteria are during the life cycle of *H. illucens* is not yet clear. To characterize and ultimately understand the functions of the present bacteria, one strategy is to qualitatively isolate strains from the GIT and assess their biochemical properties, i.e., a technique that already led to the discovery of two novel species from the GIT of *H. illucens* (45, 46). Of course, this approach is time consuming and depends on the cultivability of the isolated strains. So far, the cultivability of species identified using next-generation sequencing has not yet been addressed; thus, strains with key functions might be missed. It might be interesting in the future to use the technical advances in genomics, transcriptomics, and metabolomics to explore the functions exerted by the bacterial microbiota of *H. illucens*. Nevertheless, research in other insects already provides some potential roles that microbes might exert in *H. illucens*. So far, experimental observations are in line with these predicted functions and have confirmed the roles for bacteria in mediating oviposition, aiding food digestion, and breaking down xenobiotics (23).

Impact on oviposition. While it has been known for decades that *H. illucens* females prefer decomposing material for oviposition (47), the role of bacteria in this preference only recently came into focus (48). This kind of interkingdom interaction was already

reported for several other Diptera. For instance, gravid mosquitoes are attracted to a specific combination of 14 bacterial species (49), and gravid house flies sense volatiles produced by microbes on conspecific eggs to ensure synchronous larval development (50). Other insects can also interfere with each other's interkingdom interactions. For example, the presence of *H. illucens* larvae inhibits oviposition by the house fly (51). The most likely explanation is that the *H. illucens* larvae reduce the *Escherichia coli* numbers in the substrate (52), which is assumed to be a nutritional source for the house fly larvae (53).

Considering *H. illucens*, the presence of sterile eggs, devoid of microbes, results in less oviposition by other females than when nonsterile eggs are present (48). An assessment of the impact on oviposition of 18 bacterial species, isolated from *H. illucens* or from competing insects, illustrates the complex interplay between *H. illucens* and specific microbial species. More specifically, an aliquot of specific bacteria at a concentration of 10^4, 10^6, or 10^8 CFU per ml was put on an agar plug, which was sterilely inserted in the flutes of one of two sterilized cardboard blocks to assess the impact of their presence on the egg deposition preference. Two bacterial species, *Ignatzschineria* sp. and *Acinetobacter* sp., isolated from known competitors of *H. illucens*, the secondary screwworm (*Cochliomyia macellaria*) and the lesser mealworm (*A. diaperinus*), respectively, were found to trigger repellency and result in less egg deposition. In contrast, two other bacterial species enhance oviposition, *Gordonia* sp. (isolated from *H. illucens* eggs) and *Providencia* sp. (isolated from the hairy maggot blowfly [*Chrysomya rufifacies*]). As the latter insect preys on other blowfly species, it makes sense that there is no competition between *H. illucens* and the hairy maggot blowfly. Intriguingly, this bacterial species is also reported to be present in all life stages of *H. illucens*, adding weight to the expectation that this species plays a key role in regulating oviposition (48).

Impact on food digestion (enzymatic activities). A biochemical characterization of extracts from the GIT of *H. illucens* demonstrated high amylase, lipase, and protease activities (54). Most of these activities were very high in the guts of the larvae, while extracts from their salivary glands exhibited less than 10% of the total enzyme activity. Hence, for *H. illucens*, the gut appears to be the main site for digestion, in contrast to the situation in, for example, insects with primarily extraoral digestion (54). Furthermore, both qualitative and quantitative assays reveal that more kinds of digestive enzymes, often also with higher levels of activity, are present in the gut of *H. illucens* than in that of the house fly, which explains why the former larvae belong to the most efficient scavengers of all known species of fly (54, 55).

Gut microbes play an important role in aiding their host during the digestion of complex substrates by possessing metabolic properties that the insect lacks. Indeed, studies have shown that a number of metabolic reactions occurring in the insect GIT are encoded by the metagenome of its microbiota rather than by its own genome (56, 57). These microbial functions during digestion could be the basis of the observed substrate-dependent microbial community structure variations, as the bacteria more adapted to digest a specific substrate will have a fitness advantage in the GIT over less-adapted bacteria.

Considering the metabolic capacities of *H. illucens* larvae and the observation of the high enzymatic activity in their GITs, the bacterial communities in the larval GITs can also be mined to find novel microbial enzymes such as proteases, cellulases, lipases, xylanases and pectinases that degrade organic compounds and might be of particular interest for industrial processes. This potential is demonstrated by the identification of a novel cellulase and an alkaline amylopullulanase from the larval gut microflora using a function-based metagenome screen (24, 25). Particularly, the amylopullulanase has attractive biotechnological characteristics toward exploitation. It is active at lower temperatures than the current industrial enzymes and shows a broad pH tolerance and an increased durability toward several noxious compounds. It can be expected that in the future, more microbial enzymes will be harvested from the GITs of *H. illucens* larvae,

considering their remarkable capacity to digest a wide range of complex substrates, e.g., the digestion of lignocellulose into xylose, which enables biodiesel production (58).

Impact on detoxification of substrates. Various xenobiotics, such as antibiotics or pesticides, are complex molecules that can cause problems in the environment and for prolonged periods of time due to their high stability. Not only can *H. illucens* larvae grow in substrates contaminated with these molecules, but their presence also results in a shorter half-time of these pharmaceuticals and pesticides, e.g., 1.1 days instead of 25 days for trimethoprim (59). Hence, *H. illucens* could play a significant role not only in the recycling of biological waste but also in its bioremediation into a safer composted material for reuse in the environment. Data suggest that in the house fly, the GIT microbiota aids in the degradation of such molecules by harboring specific metabolic pathways (60). Also, in pest insects, symbioses with microorganisms have been identified that enable the degradation of complex plant allelochemicals and xenobiotic insecticides (61). Therefore, the *H. illucens*-associated microbiota most likely also facilitates the observed degradation, maybe even in a faster way than the house fly. In the future, it will be interesting to mine the metagenome of this microbiota to identify the xenobiotic degradation mechanisms and exploit those enzymes for bioremediation applications.

A category of natural toxic molecules is the mycotoxins, such as aflatoxin B1 (AFB1), which are produced by fungi that grow on specific crops (such as cereals or nuts). The contaminated crops are often removed from the food chain. However, *H. illucens* larvae can grow on substrates containing up to 0.415 mg/kg of AFB1 without showing accumulation. In fact, AFB1 appears to be catabolized or at least bound to proteins (62). *H. illucens* larvae could thus be used to recycle mycotoxin-contaminated substrates. The question remains as to whether the microbiota plays a role in this catabolism.

IMPACT OF MICROBIAL COMMUNITY MANIPULATION ON *HERMETIA ILLUCENS*

The microbiota of *H. illucens* forms a dynamic complex community that serves a role in various aspects during its life cycle. As a consequence, its composition is an important parameter to consider when optimizing the industrial rearing process. A well-established strategy to investigate how microbes influence the growth of an insect involves the administration of specific bacteria to the insect directly or to the substrate and then assessing their impact on growth and microbial dynamics. This method is referred to as "microbial challenging." The outcome of challenging insects with bacteria of course depends on the selected bacterial species. In this regard, two major types can be distinguished. On one hand, bacteria can be investigated with the aim to enhance insect growth, e.g., by improving substrate digestibility. On the other hand, the transmission of pathogens, either entomopathogens or pathogens for downstream organisms, from the substrate to the larvae can be studied.

Improvement of growth performance by adding companion bacteria. For several farm animals, a well-defined set of probiotics has already been developed and is commercially available. Probiotics are live microorganisms which confer a health benefit for the host when administered in appropriate and regular quantities (18). For insects used in industrial rearing, this is virtually uncharted terrain. Nevertheless, Yu et al. (63) reported in 2011 the potential for the use of so-called companion bacteria. Four different *Bacillus* strains, three isolated from the larval gut (*B. subtilis* S15, S16m, and S19) and one from the feed (*B. subtilis* subsp. *natto*), were shown to enhance the growth of the larvae. This is most likely due to their aid in the digestion of the provided substrate (chicken manure). Treatment with each of the strains resulted in heavier larvae combined with a shortened development time, going from 34.33 $\pm$ 3.51 days between hatching and 90% reaching the prepupal stage for the uninoculated sample to 29.00 $\pm$ 1.00 days for the samples inoculated with the *B. subtilis* S15 strain (63). This reduced development time can directly impact the productivity of *H. illucens* as an insect for industrial rearing. However, with the reported error margins, it remains to be seen if this effect would yield an economic benefit. At the same time, it might be that other bacteria will have a more pronounced effect under specific conditions. Future

TABLE 2 Results of published studies using challenging with pathogenic bacteria

Study authors and year of publication	Substrate	Storage	Incubation (h)	Microorganism	Inoculation (CFU/g)[a]	Temp (°C)	Reduction (log_{10} CFU/g)[a]
Erickson et al., 2004 (64)	Cow manure	1 day at −20°C	72	*E. coli*	10^7	27	≈0.5
	Hog manure						≈3.5
	Chicken manure	1 day at −20°C	72	*Salmonella*	10^7	27	≈2.5[b]
				E. coli		23	≈1.0
				E. coli		27	≈6.0[b]
				E. coli		32	≈6.0[b]
	Chicken manure	45 min at 121°C	72	*Salmonella*	10^7	27	≈2.5[b]
Liu et al., 2008 (52)	Dairy manure	7 days at −20°C	72	*E. coli*	10^7	23	5.1[b]
						27	6.1[b]
						31	5.0[b]
Lalander et al., 2013 (65)	Human feces	−20°C	192	*Salmonella*	10^7	20–25	6.0[b]
				Enterococcus	10^6		<1.0[b]

[a]≈, values were derived from the graphs in the article.
[b]Statistically significant reduction compared to the controls in the study.

efforts may aim to identify novel strains with even more pronounced beneficial effects, which can be matched with specific substrates, and to commercialize them for the insect industry.

Reduction of specific pathogens administered in the substrate. To maximize the sustainable nature of *H. illucens* as an alternative protein source, it is envisioned that in the future, these insects will be industrially grown on various waste streams, probably even in different stages of decay or including animal or human manure, which is currently not allowed in the EU (EG no. 767/2009). Such substrates are an ideal reservoir for many significant foodborne pathogens, including but not limited to *Escherichia coli* O157:H7 and *Salmonella* spp. Therefore, it is of key importance to assess how their abundance is affected by the presence of *H. illucens* larvae to ensure that pathogens do not accumulate in the larvae nor the final product. So far, a few studies focused on the interactions between the larvae and the two mentioned pathogens and found that the bacterial counts in the substrates for both pathogens were reduced (Table 2) and that neither of their numbers accumulated inside the larval body (52, 64, 65).

Furthermore, it appears that the effectiveness of pathogen reduction depends on the temperature and the composition of the substrate. Indeed, *E. coli* O157:H7 numbers were reduced more effectively at a temperature between 27°C and 32°C than at 23°C (64). At a higher temperature (35°C), substrate-dependent reductions in bacterial counts were observed, even in the controls not containing *H. illucens*, as well as 100% mortality of the larvae (52). An example of the effect of the substrate composition is the difference in the reduction of fluorescently labeled *E. coli* O157:H7 cells in chicken manure compared to that in hog manure. In chicken manure, the presence of *H. illucens* larvae led to an increased level of reduction in the *E. coli* O157:H7 population, while in the hog manure, the observed pathogen reduction was the same whether the larvae were present or not. A difference between these two manures that might play a role in the missing antimicrobial activity of the larvae in the hog manure is their pH. The hog manure had an initial pH of 6.0 to 6.2 that decreased over time, while the chicken manure had an initial pH of 7.4 to 8.2 that increased over time (64). The growth of the larvae was not influenced by this lower pH, which matches with the claims of industrial rearers that larvae grow on fermented substrates (personal communications). In fact, larval growth was more than double in the hog manure compared to that in the chicken manure; thus, the reduced growth cannot explain the absence of pathogen reduction. The authors pose as a possible explanation that the antimicrobial compounds present in *H. illucens* larvae are less stable or active at the lower pH values (64). For example, this is the case for a known insect-harbored antibacterial protein, defensin A, of the flesh fly, *Phormia terranovae*, which has a maximal stability at a pH of around 8.3 (66).

In conclusion, the antibacterial mechanisms of *H. illucens* larvae seem to be able to differentiate between organisms, as they do not target all bacteria. As stated, specific strains of *Salmonella* and *E. coli* are clearly targeted (64). Indeed, Lalander et al. (65) also observed a quick drop below detection levels of a strain of *Salmonella enterica* in the GITs of human-manure-fed larvae. Interestingly, the counts of another tested pathogen, *Enterococcus* spp., were unaffected for more than 8 days by larval presence (65). These observations illustrate that *H. illucens* larvae are able to reduce the load of specific pathogens in their substrates, under the right conditions, without themselves accumulating these pathogens. However, the studies performed so far do not indicate a complete eradication of pathogens. Therefore, a processing step with a decontaminating effect is still recommended prior to the use of larvae as a feed ingredient.

EXPLOITATION OF ANTIBACTERIAL PROPERTIES OF *HERMETIA ILLUCENS*

Over the past decades, a rapid increase in the emergence of multidrug-resistant bacteria has been observed, while drug discovery is struggling to keep up with delivering novel antibiotics to combat these "superbugs" (e.g., ESKAPE [*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species] bacteria *P. aeruginosa* or *Enterobacter* species). To prevent a return to the preantibiotic era, new antibacterials have to be developed and the excessive use of existing antibiotics must be avoided. This has led to a total ban on the use of antimicrobial growth promoters (AGPs) in livestock feed in many countries across Europe (regulation EC/1831/2003) and the United States (Veterinary Feed Directive). With additional restrictions on the use of zinc oxide at pharmacological doses in the EU upcoming in the near future (67), finding novel and effective replacement strategies to manipulate and control the animal GIT microbiota is a high priority.

Taking advantage of the robust immune systems of the *H. illucens* larvae has recently gained attention as a promising approach to fulfill this need. In *H. illucens*, immunity is a combination of cell-mediated and humoral innate mechanisms. Hemolymph cells, of which *H. illucens* was found to harbor three types (68), are the main actors in the cellular immune responses, e.g. in phagocytosis, nodulation and encapsulation, while phenoloxidase, antimicrobial peptides (AMPs), and proteins (e.g., lysozymes) are a few examples of key components of the humoral innate response.

Several strategies are now being investigated to exploit to the fullest the potential of *H. illucens* larvae immunity. One strategy explores whether the antibacterial properties of *H. illucens* larvae can impact the health of farm animals, when these are fed with the whole insects. With this in mind, techniques to augment or direct the antibacterial activity of these insects could deliver an even superior in-feed antibacterial additive, and these are also explored. While the direct use of *H. illucens* larvae would be straightforward, it does require that the antibacterial molecules remain stable during feed processing and digestion. To exploit those antibacterial molecules that do not meet these stability requirements, another strategy aims to extract and characterize these molecules and use them as inspiration for the design of novel more stable antibiotics. In the next paragraphs, the current state of the art of these strategies will be discussed.

Use of larvae as a novel feed additive to aid host immunity. If the antibacterial activities observed in *H. illucens* larvae could persist in the farm animals fed with these larvae, then these larvae could be used as an alternative to the AGPs used in the livestock industry in the past to improve gut health. Unfortunately, the effect on animal health of *H. illucens* larvae has hardly been investigated. So far, the focus has been on the nutritional impact of feeding insects to other animals (69–72). Recently, Spranghers et al. considered gut health parameters of weaned piglets that were fed with diets containing either defatted or full-fat BSF prepupae (73). They reasoned that the high content of lauric acid ($C_{12:0}$), known for its antimicrobial effects against Gram-positive bacteria, in the fat of the larvae could result in antimicrobial effects in the piglet gut and provide added value to *H. illucens* larvae as a protein source. While they observed a

0.5-log reduction in group D streptococci counts in the gut, the decrease was not statistically significant. A possible explanation for this weak impact is that only 0.03 g $C_{12:0}$/100 g of the total 1.58 g $C_{12:0}$/100 g present in the feed reached the gut, meaning that most of the $C_{12:0}$ was absorbed earlier in the stomach (73). What could boost the efficacy of *H. illucens* larvae as an antimicrobial feed additive is high levels of other components of the insect humoral response (e.g., AMPs). Unfortunately, the levels of these components were not specifically investigated in this study. However, the authors stated the number of AMPs was expected to be low, and it is also not known if AMPs remain stable during feed processing and are able to reach the gut. In the future, this stability will have to be studied to achieve a successful implementation of whole *H. illucens* larvae as an antibacterial feed additive.

Modulation of larval immunity to improve their antibacterial properties. Alternatively, the antibacterial efficacy of the *H. illucens* larvae can be increased by developing methods to boost their immune systems, compensating for the losses in activity during processing. To achieve this, it is necessary to know how the expression of the immunity actors in the larvae is regulated and whether immunity can be directed to target specific pathogens. Recent research, focusing on the immune mechanisms of *H. illucens* and the modulation thereof, has shown that its immunity is indeed inducible, increasing the feasibility for the exploitation of *H. illucens* larvae as an antibacterial feed additive (68). Challenging the immunity of *H. illucens* larvae by introducing bacteria via piercing was found to increase phenoloxidase activity in the hemolymph and to induce the production of AMPs (68). As a result, the antimicrobial activity of the hemolymph increased, even in a pathogen-specific manner. Indeed, larvae challenged with the Gram-positive *Micrococcus luteus* produced no activity toward the Gram-negative *E. coli*. On the other hand, larvae challenged with *E. coli* did generate activity against Gram-negative bacteria and also produced a higher activity against Gram-positive bacteria (68). This suggests a discrimination between Gram-positive and Gram-negative bacteria by the immune system. This distinct immunity response is related to the production of two proteins of approximately 58 kDa and 27 kDa in the hemolymph of the *E. coli*-challenged larvae, while two other proteins of approximately 3.8 kDa and 43 kDa are expressed after both challenges (68). The authors proposed that by varying the bacteria used for challenging, different sets of antimicrobial compounds will be induced. While the piercing method could improve the antibacterial properties of *H. illucens* larvae in feed, it is not feasible at an industrial scale.

A more achievable approach was recently demonstrated, which involves the substrate-dependent alteration of *H. illucens* immunity (74). The manipulation of larval immunity via the substrate composition is an example of the emerging field of nutritional immunology. In this study, larvae were fed a standard diet supplemented with a mixture of bacteria or with different additives of organic waste, such as sulfonated lignin and sunflower oil. Next, the antimicrobial activity of larval extracts and the immunity-related transcriptome were analyzed. The transcriptome analyses revealed that *H. illucens* harbors a more diverse spectrum of AMP and lysozyme gene family members than most insects, containing 6 attacins, 7 cecropins, 26 defensins, 10 diptericins, and 4 knottin-like peptides (74). Surprisingly, the protein-rich diet (supplemented with brewer's grains) triggered the most differential expression of these genes, followed by the plant oil diet. Both diets induced stronger immune responses than when bacteria were directly added to the diet. Nevertheless, the supplementation of bacteria also triggered a significant upregulation of more than half of the identified candidate AMP genes (74). On the basis of growth inhibition assays, the strongest inhibition of Gram-negative bacteria was observed with the aqueous extracts from the larvae reared on the high-protein and cellulose diet, while for Gram-positive bacteria, the most active extracts were those from the larvae reared on chitin, cellulose, bacteria, and plant oil (Table 3) (74). This diet-dependent expression of AMPs most likely enables *H. illucens* larvae to adapt the microbiome from the substrate as well as the host-associated core microbiome to enable a flexible digestion of a variety of diets. Future

TABLE 3 Impact of additives on antimicrobial properties in extracts of *H. illucens* larvae[a]

Additive	Extraction solvent	More active than extracts from control diet?[b]			
		E. coli	*M. luteus*	*P. fluorescens*	*B. subtilis*
High protein	Water	No	No	Yes	Yes
	Chloroform	No	No	No	No
Chitin	Water	No	Yes	No	Yes
	Chloroform	No	No	No	No
Lignin	Water	ND[c]	ND	No	No
	Chloroform	Yes	Yes	No	Yes
Cellulose	Water	No	Yes	Yes	Yes
	Chloroform	No	No	No	No
Plant oil	Water	No	Yes	No	Yes
	Chloroform	Yes	Yes	Yes	Yes
Bacteria	Water	No	Yes	No	No
	Chloroform	Yes	Yes	Yes	Yes

[a]As determined from Vogel et al., 2018 (74).
[b]Determined by disk diffusion antimicrobial assays.
[c]ND, not determined.

research will have to explore if the expression of specific compounds is responsible for the selection of a unique microbial community in response to environmental factors in *H. illucens*.

The characterization of the underlying mechanisms behind these diet-dependent expressions would enable the exploitation of this immunity activation and/or modulation, increasing the potential of *H. illucens* as a viable alternative to antibiotics in feed. On the basis of the substrate the larvae are fed, they can then be directed to express specific immunity genes enabling the control of infectious pathogens in and safeguarding the health of a wide array of livestock and companion animal species.

Search for antibacterial compounds expressed by the larvae or their microbial community. To circumvent the problems associated with molecules having to withstand processing and digestion, antibacterial molecules can be purified and identified directly from larval extracts, or alternatively, selected immunity-related genes of *H. illucens* can be expressed *in vitro* and characterized. Both approaches aim to discover novel molecules that could serve as the inspiration for the design of novel drugs.

Several studies examined the bioactive effects of different larval extracts (74–77). These studies show various antimicrobial activities against different bacteria that depend on the environment in which the larvae were reared, as well as the extraction method used (see Table S1 in the supplemental material). Their activity was also able to hinder the early stages of biofilm formation, which is a logical consequence of their antibacterial activity against planktonic cells (76). So far, no one has investigated whether these extracts also can eradicate mature biofilms. To identify bioactive molecules from the crude extracts, further separation methods (e.g., Sep-Pak C_{18} or high-pressure liquid chromatography [HPLC]) can be applied until a pure fraction of the active compound is obtained for identification. Using this strategy, Park et al. (77) showed that the compound responsible for activity against *E. coli* was strongly hydrophilic and that several compounds with strong activity against methicillin-resistant *Staphylococcus aureus* (MRSA) act in synergy. Furthermore, these compounds are highly robust, as they remained active after several freeze-thaw cycles and after lyophilization. Such parameters are key properties for a stable pharmaceutical product. The identified molecule was named DLP4, a defensin-like peptide with a potent activity against Gram-positive bacteria (78). Using a similar approach, hexanedioic acid was also identified as a new substance with antibacterial activity against various pathogenic bacteria, including MRSA (79).

TABLE 4 Characterized AMPs of *H. illucens* to date

Study authors and year of publication	Peptide			Pathogen tested	Active?	MIC
	Name	Size (aa)	Class			
Park et al., 2015 (78)	DLP4	47	Defensin	*E. coli*	No	ND[a]
				Enterobacter aerogenes	No	ND
				P. aeruginosa	No	ND
				MRSA	Yes	0.59–1.17 μM
				S. aureus 40881	Yes	0.59–1.17 μM
				S. aureus 12256	Yes	1.17–2.34 μM
				B. subtilis	Yes	0.59–1.17 μM
				Staphylococcus epidermis	Yes	0.02–0.04 μM
Elhag et al., 2017 (80)	Trx-stomoxynZH1	63	Cecropin[b]	*E. coli*	Yes	15–30 μg/ml
				S. aureus	Yes	27–54 μg/ml
				Rhizoctonia solani	Yes	>98 μg/ml
				Sclerotinia sclerotiorum	Yes	>98 μg/ml
Li et al., 2017 (81)	DLP2	40	Defensin	*S. aureus*	Yes	0.01–0.23 μM
				Streptococcus suis	Yes	0.93 μM
				Listeria ivanovii	Yes	0.12 μM
				E. coli	No	>29.97 μM
				Salmonella enterica serovar Typhimurium	No	>29.97 μM
				S. enterica serovar Enteritidis	No	>29.97 μM
	DLP4	40	Defensin	*S. aureus*	Yes	0.01–0.47 μM
				S. suis	Yes	4.0 μM
				Listeria ivanovii	Yes	0.12 μM
				E. coli	No	>29.98 μM
				S. Typhimurium	No	>29.98 μM
				S. Enteritidis	No	>29.98 μM

[a]ND, not determined.
[b]Fusion protein.

Recently, the technological advances in genomics and transcriptomics have enabled a more direct approach for the identification and characterization of immunity genes. For AMPs, several online databases have emerged, which contain over 2,000 molecules. Comparing new sequences to such databases enables the rapid identification of candidate AMPs in organisms. Using a reference set of insect-derived AMPs and lysozymes, a total of 53 genes were annotated as putative AMPs in *H. illucens* (74). The challenge now is to express such genes and characterize their antibacterial properties. So far, a total of 9 AMPs are reported to be cloned, expressed, and structurally analyzed. For three of these, the MIC values against various strains were determined (Table 4) (80, 81). The first is stomoxynZH1, an AMP of the class of the cecropins, which consists of a linear cysteine-free peptide of 60 amino acids (aa) with an alpha helix-like structure. Studies with homologous stomoxyns showed that this molecule has a broad activity spectrum, with low cytotoxicity and a rapid activity upon exposure. The *H. illucens*-derived molecule, fused to thioredoxin, also had both antibacterial and antifungal activities. In the future, the stability of this peptide without the fusion needs to be investigated (80).

Two other peptides that were characterized are DLP2 and DLP4. These homologous defensin-like peptides have high sequence similarities (60 to 65%) to other known insect defensins: sapecin, insect defensin A, and lucifensin. These molecules are potent antibacterials against Gram-positive bacteria, with DLP4 killing bacteria after just 0.5 h of exposure. On the other hand, they have almost no impact on Gram-negative bacteria. DLP2 and DLP4 retain 85% and 93%, respectively, of their activity after an incubation of 1 h at 80°C, which shows a high thermal stability. Furthermore, the observed cytotoxicity toward eukaryotic cells is low for both molecules. MRSA bacteria also fail to develop additional resistance during 30 serial passages, while they do gain resistance toward other commercially used antibiotics in that time frame. To summarize, *in vitro*, these two peptides entail a high stability, have no impact on eukaryotic cells, and trigger low rates of bacterial resistance development, which makes them

promising drug candidates. However, these *in vitro* conditions obviously differ greatly from those in clinical use. To this end, 6-week-old female BALB/c mice were injected intraperitoneally with MRSA on one side and two doses of the DLP on the other side to assess the *in vivo* potential of these novel antibacterial compounds. In mice, both peptides could not only prevent mortality, but they also triggered immunomodulatory effects that resulted in an improved balance between proinflammatory and anti-inflammatory cytokines upon bacterial infection (81). To date, this is the most comprehensive evaluation of an *H. illucens*-derived AMP toward antibiotic development. The results clearly show their potential as an innovative new class of antibacterial compounds. It can be expected that in the future, all *H. illucens* AMPs will be systematically characterized using a streamlined methodology to rapidly compare and score antibacterial potential.

CONCLUSIONS AND PERSPECTIVES

The dire need for more sustainable alternative sources of protein and the interesting characteristics insects offer in this regard are fueling the enthusiasm to rear these organisms on an industrial scale. One of the most promising species being reared is the black soldier fly (*H. illucens*). Its larvae are able to process a wide range of organic substrates and to turn them into high-quality protein that can be used as feed, while other compounds (fat and chitin) can be used as a raw material in the chemical industry (11, 12). In light of recent legislative advances, which created a legal framework and allowed access to new markets for insects as feed components, it is no longer a question of whether an entire food value chain will arise for the industrial rearing of *H. illucens* but how fast it will be installed. Awaiting this (r)evolution, it is up to researchers to gather the same level of in-depth knowledge that is available for other farm animals to support this novel industry.

It is clear that the functions of the microbial communities in *H. illucens* and their dynamics on the basis of the rearing parameters form a key piece of this puzzle. While the initial observations in this emerging field illustrate the important role that microorganisms play during *H. illucens* development, they also reveal a much broader impact this research can have aside from the development of a more efficient industrial rearing process. In general, the potential of this research field can be divided into two main categories: (i) the manipulation of the microbiota to optimize insect biomass production and (ii) the exploitation of the *H. illucens*-microbiota interplay for the discovery of new biotechnological tools.

(i) A profound understanding of the substrate-dependent alterations in the microbial community will enable us to define a substrate-dependent optimal GIT microbiota that can then be added to ensure a high feed efficiency and thus larval mass gain. On the other hand, manipulations of the microbiota can also aim to improve other characteristics of these larvae to increase their economic value. This will be an important evolution to ensure the economic competiveness of this industry toward both established and starting producers of protein sources for feed. The characteristics that one can envision to be altered entail antimicrobial activity, vitamin content, digestibility, and others. (ii) Exploring the roles that microbes play in the metabolic capacities of the *H. illucens* larvae and the mechanisms the insects' immune systems use to control the compositions of their microbial communities will yield a wealth of novel enzymes and antimicrobial molecules. Such enzymes can be superior to currently used enzymes, as already demonstrated, or be able to catalyze novel reactions. On the other hand, the antimicrobial compounds could lead to the development of *H. illucens*-derived strategies as in-feed antimicrobials, which are highly needed. Hence, this insect and its microbiota can be a rich source in the search for novel enzymes that can be exploited in bioremediation, food industry, pharma or other industries.

The next decade promises to be extremely interesting for this research field, and it will be interesting to see the further developments toward the microbiological optimization of *H. illucens* as a sustainable insect for industrial rearing and the exploitation of its microbiome for novel biotechnological applications.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02722-17.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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