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Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*

Heiko Vogel [a], Ariane Müller [b], David G. Heckel [a], Herwig Gutzeit [b], Andreas Vilcinskas [c, d, *]

[a] Entomology Department, Max-Planck Institute for Chemical Ecology, Hans-Knoell Strasse 8, 07749 Jena, Germany
[b] Institute of Zoology and Developmental Biology, University of Dresden, 01062 Dresden, Germany
[c] Institute for Insect Biotechnology, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany
[d] Department Bioresources, Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Winchesterstrasse 2, 35394 Giessen, Germany

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ABSTRACT

The black soldier fly *Hermetia illucens* is used for the bioconversion of organic waste into feed for livestock and aquaculture, and is economically among the most important farmed insects in the world. The larvae can be fed on agricultural waste and even liquid manure, resulting in highly unpredictable pathogen levels and dietary conditions. Here we show that *H. illucens* larvae express a remarkably expanded spectrum of antimicrobial peptides (AMPs), many of which are induced by feeding on a diet containing high bacterial loads. The addition of sulfonated lignin, cellulose, chitin, brewer's grains or sunflower oil revealed the diet-dependent expression profiles of AMPs in the larvae. The highest number of AMPs and the highest levels of AMP expression were induced by feeding larvae on diets supplemented with protein or sunflower oil. Strikingly, the diet-dependent expression of AMPs translated into diet-dependent profiles of inhibitory activities against a spectrum of bacteria, providing an intriguing example for the emerging field of nutritional immunology. We postulate that the fine-tuned expression of the expanded AMP repertoire mediates the adaptation of the gut microbiota to the digestion of unusual diets, and this feature could facilitate the use of *H. illucens* for the bioconversion of organic waste.

1. Introduction

The growing human population requires a corresponding increase in the food supply, and food security has therefore become the top priority among the sustainable development goals for a global bioeconomy. More efficient animal production, meat substitutes and alternative protein sources are required to avoid the predicted protein gap (El-Chichakli et al., 2016). In this context, insects are highly efficient converters of organic waste into feed for livestock or aquaculture. The Food and Agricultural Organization of the United Nations (FAO) has promoted insects as a high-quality, cost-effective, energy-efficient and sustainable alternative protein source since 2003. The black soldier fly *Hermetia illucens* has emerged as the most important insect in the world for bioconversion because the larvae can feed on virtually all types of organic waste, including used frying oil, lignin, cellulose and liquid manure (Müller et al., 2017). Accordingly, they are becoming increasingly prevalent as a means for protein production and waste management, and several companies focus on the farming of this species on the multi-ton scale (Makkara et al., 2014; Nguyen et al., 2015).

The ability of insects to survive and feed on diets that are nutritionally unpredictable or often contaminated with high bacterial loads is thought to be mediated at least in part by corresponding adaptations in the immune system (Vilcinskas, 2013). Recent studies provide evidence that immunity-related effector molecules such as antimicrobial peptides (AMPs) are not only expressed constitutively or when immune responses are triggered by invading pathogens, but also in an organ, gender and environment dependent context (Vogel et al., 2017). For example, the expression of particular AMP subsets in the burying beetle *Nicrophorus vespilloides* is gender-specific and regulated by the presence

* Corresponding author. Institute for Insect Biotechnology, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany.
E-mail address: andreas.vilcinskas@agrar.uni-giessen.de (A. Vilcinskas).

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of offspring or carcass (Jacobs et al., 2016). This observation inspired us to explore how diet influences insect immunity. To contribute to the emerging field of nutritional immunology (Ponton et al., 2011, 2013), we selected the omnivorous larvae of *H. illucens* as a model. To test the impact of variable diets in combination with the developmental stage of larvae on the expression of immunity-related genes, we fed the larvae on diets supplemented with a mixture of bacteria as well as with different forms of organic waste: sulfonated lignin, cellulose, chitin, brewer's grains (protein-rich diet), sunflower oil and bacteria-enriched diet. We isolated total RNA from whole larvae as well as the larval midgut, hindgut and salivary glands and analyzed the immunity-related transcriptome by next-generation sequencing as previously described (Pöppel et al., 2015; Vogel et al., 2011). We focused on the identification and relative expression levels of immunity-related genes encoding pathogen recognition receptors (PRRs), phenoloxidases, lysozymes and AMPs, looking in particular for evidence of AMP gene family diversification and their diet-dependent expression. In addition, we screened the extracts obtained from larvae fed with different diets against Gram-positive bacteria (*Bacillus subtilis* and *Micrococcus luteus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas fluorescens*). Strikingly, we observed both the diet-dependent expression of AMP subsets and diet-dependent profiles of antibacterial activity.

2. Material and methods

2.1. Insect rearing and treatment

Larvae of the black soldier fly (*Hermetia illucens*) were obtained from our rearing pilot plant in Grimma, Saxony, Germany, and were maintained at 24 °C in the laboratory. We prepared 10 g of feed per 10 g of larvae and per diet. A total of eight different diets were prepared for larval rearing. The standard diet was 10 g of normal feed (deuka all-mash L gekörnt) plus 10 ml water. Supplemented diets were prepared by mixing 5 g normal feed with 5 g of the following supplements plus 10 ml water: Lignobond DD sulfonated lignin (Vilomix), alphacel cellulose powder (MP Biomedicals), chitin (poly-(1 → 4)-β-*N*-acetyl-D-glucosamine; Sigma), brewer's grains (Waldschlösschen Brewery Dresden) or sunflower oil (Thomy). Furthermore, we prepared a mixed diet by combining 50 g normal feed (standard diet) with 10 g each of cellulose, chitin, lignin, brewer's grains, and sunflower oil, and 50 g of a food waste mix (50% w/w plant and meat mix). To elicit an immune response, the standard diet was supplemented with 1.25 g of each bacterial strain (see below for details on bacterial species used). Abbreviations of the eight different diets were: Con = control diet (standard diet), HBact = high bacterial load (bacteria-mix added to standard diet), Cell = cellulose, Chit = chitin, Lig = lignin, ProtR = high protein (brewer's grains), POil = plant oil, Mix = mixed diet. Larvae were reared on the respective diets for 72 h at 24 °C. Extracts for testing antimicrobial activity were prepared by washing the larvae with water containing disinfectant, rinsing with sterile water, drying with tissue paper, freeze/thawing at −20 °C and grinding thoroughly in methanol/water/acetic acid (90/9/1 v/v/v). The extract was centrifuged at 1600 × g for 20 min at 4 °C and the methanol removed in a rotary evaporator under reduced pressure. Proteins and lipids were isolated from the sample by sequential extraction with chloroform and ethyl acetate according to Park et al., (2014). The three samples per diet were concentrated in a rotary evaporator under reduced pressure and stored in a refrigerator at 4 °C.

2.2. Microorganisms and inhibition zone assay

Standard plate-growth inhibition zone assays were carried out with the bacterial species *Escherichia coli* BL21 (DE3), *Micrococcus luteus*, *Pseudomonas fluorescens* BL915 and *Bacillus subtilis*, kindly provided by the Institute of General Biochemistry, TU Dresden. The bacteria were cultivated in lysogeny broth at 30 °C, shaking at 150 rpm. Petri dishes (150 × 20 mm) were prepared with lysogeny broth containing 1.5% agarose. The agar base was coated with 15 ml of a top agar layer containing lysogeny broth with 0.8% agarose and 900 µl of the bacterial suspension (OD_{600} = 0.5). Rotilabo® test flakes (6 mm diameter, Carl Roth GmbH) were loaded with the relevant extracts or controls (methanol, chloroform, ethyl acetate, 2.88 mg/ml chloramphenicol) and placed on the top agar layer. After overnight incubation at 30 °C, the diameters of the clear zones were measured. All experiments were performed in triplicate.

2.3. RNA-seq, de novo assembly and candidate gene identification

After brief washes in PBS and ethanol, two pools (biological replicates) of five *H. illucens* larvae for each of the seven diet treatment groups were shock-frozen for RNA isolation. For the larvae reared on a mixed diet, 20 larvae were briefly washed in PBS and ethanol. Ten larvae were shock-frozen for RNA isolation and the remaining larvae were dissected in ice-cold PBS, and the foregut/midgut, hindgut and salivary glands were submerged in lysis buffer and shock-frozen. RNA was isolated using the innuPREP RNA Mini Kit (Analytik Jena AG) according to the manufacturer's instructions. Transcriptome sequencing was carried out by GATC Biotech on an Illumina HiSeq2500 Genome Analyzer platform, using paired-end (2 × 100 bp) read technology for the 14 whole-larvae diet samples and single read (1 × 100 bp) technology for the mixed larval diet and tissue samples, yielding approximately 30–40 million reads for each sample. Quality control measures and *de novo* transcriptome assembly, combining all RNA-Seq samples, were carried out using CLC Genomics Workbench v8.1 (http://www.clcbio.com) as previously described (Vogel et al., 2011). The *de novo* reference transcriptome assembly (backbone) of *H. illucens* contained 25,197 contigs (minimum contig size = 250 bp) with an N50 contig size of 1330 bp and a maximum contig length of 23,709 bp. Transcriptome annotation using BLAST, Gene Ontology and InterProScan was carried out as previously described (Ponton et al., 2011; Pöppel et al., 2015). *H. illucens* candidate AMP genes were identified using an established reference set of insect-derived AMPs and lysozymes and additional filtering steps as previously described, to avoid interpreting incomplete genes or allelic variants as further AMP genes (Jacobs et al., 2016; Vogel et al., 2014). The predicted AMP and lysozyme sequences are listed in Table S1. The short read data have been deposited in the EBI short read archive (SRA) with the following sample accession numbers: ERS1508397-ERS1508410. The complete study can also be accessed directly using the following URL: http://www.ebi.ac.uk/ena/data/view/PRJEB19091.

2.4. Mapping and differential gene expression analysis

Digital gene expression analysis was carried out using CLC Genomics workbench v8.1 to generate BAM mapping files, and finally by counting the sequences to estimate expression levels, using previously described parameters for read mapping and normalization (Jacobs et al., 2016; Vogel et al., 2014). To control for the effect of global normalization using the RPKM method (normalized mapped read values based on geometric means of the biological replicate samples), we analyzed a number of highly-conserved housekeeping genes, including those encoding GAPDH, ribosomal

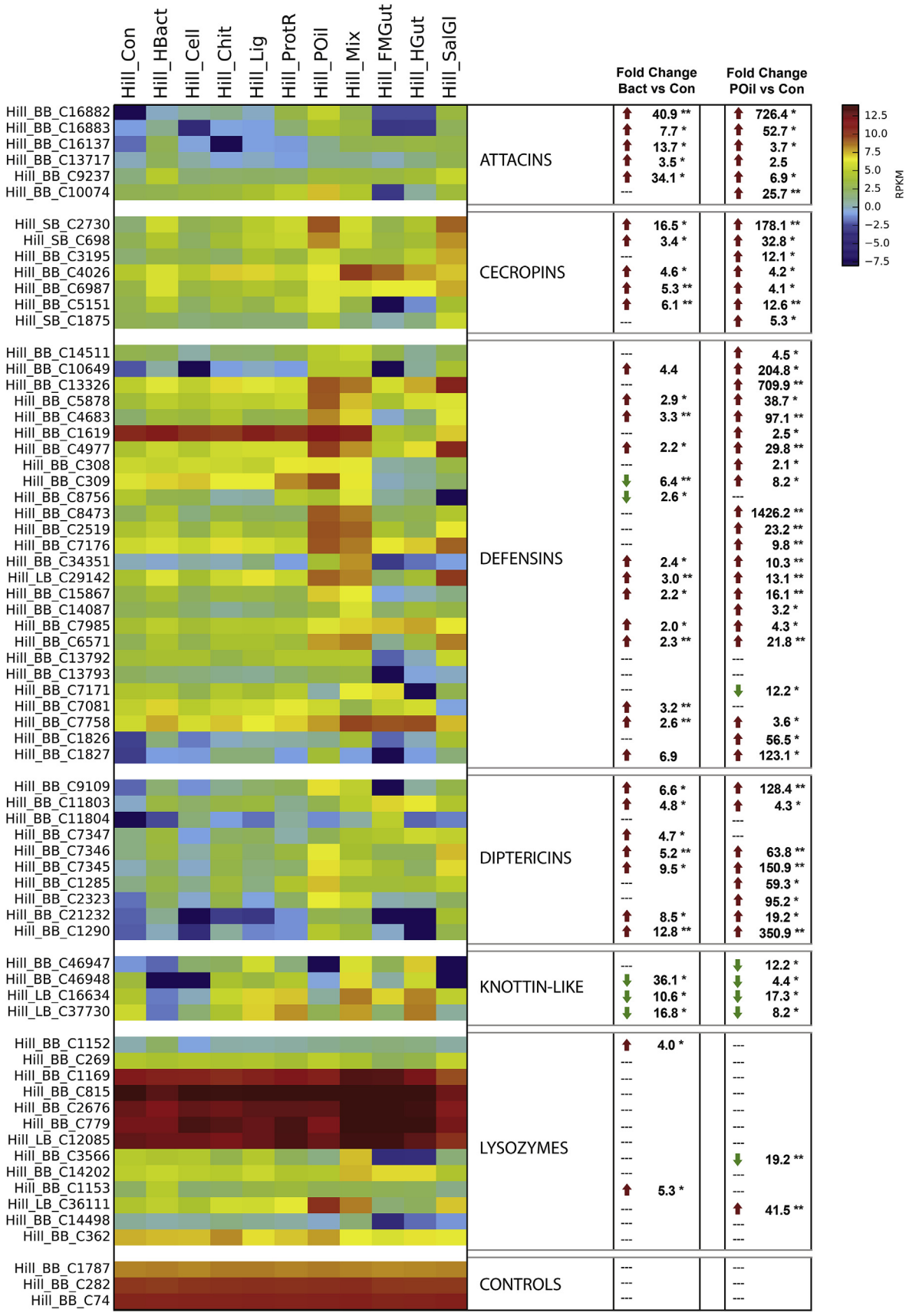

Fig. 1. Heat map showing the relative expression levels of AMPs and lysozymes in *H. illucens* larvae in relation to different diets and tissues. Fold change gene expression differences among larvae exposed to high bacterial loads and plant oil-rich diets are compared to controls on the right. Red arrows indicate upregulation and green arrows indicate down-regulation of the corresponding contigs. Significant differences (FDR-corrected p-values) are shown with an asterisk (* < 0.05, ** < 0.005). Genes of interest corresponding to the lysozyme and AMP families are grouped. The housekeeping genes *GAPDH*, *RPS4e* and *EIF5a* are used for normalization and are shown to confirm the uniform expression of these

protein Rps4e and eukaryotic translation initiation factor 5a. The overall expression level variation for these housekeeping genes was lower than 1.2-fold, indicating they were not differentially expressed. In addition to the method implemented in Qseq, we used an alternative method for normalization and differential gene expression analysis. Mapped reads were log2-transformed and normalized using the quantile method and statistical analysis of the normalized data was carried out using the "empirical analysis of digital gene expression" (EDGE) tool, implemented in CLC Genomics Workbench v8.1. For both methods, the thresholds for differentially expressed genes were a minimum two-fold change in expression and a false discovery rate (FDR)-corrected p-value of <0.05.

3. Results

3.1. Identification and relative expression levels of immunity-related genes

The screening of our *H. illucens* larval *de novo* transcriptome assembly revealed a diverse spectrum of AMP and lysozyme gene family members. The number of predicted AMPs belonging to different families was higher than in most other insects, with 6 attacins, 7 cecropins, 26 defensins, 10 diptericins and 4 knottin-like peptides. We identified a total of 53 genes encoding putative AMPs in the *H. illucens* transcriptome (Fig. 1). We also identified genes mediating the recognition of bacteria, such as those encoding peptidoglycan recognition proteins (PGRPs), Gram-negative bacteria binding proteins (GNBPS) and phenoloxidases (Fig. 2).

Principal component analysis of the *H. illucens* RNA-Seq data (Fig. S1) indicated good replicate reproducibility and striking differences between individual diets. The largest number of significantly differentially expressed genes compared to the control diet was observed in the protein-rich diet (supplemented with brewer's grains) followed by the plant oil diet, whereas the lowest number was found in the larvae exposed to cellulose (Fig. 3). Similarly, the relative expression levels of individual genes encoding PGRPs, GNBPs, phenoloxidases, lysozymes and AMPs appeared to be determined by the diet (Fig. 2 and Fig S1) but there was also some evidence for tissue-specific expression. Several lysozymes were strongly expressed in a constitutive manner and were not affected by the diet. Although the presence of a large bacterial load in the diet resulted in the significant upregulation of more than half of the identified candidate AMP genes, larvae fed on the diet supplemented with plant oil displayed the most robust overall induction of immunity-related genes, both quantitatively and qualitatively (Fig. 1).

3.2. Diet dependent antibacterial activities in H. illucens

Standard plate-growth inhibition assays were used for the relative quantification of the diet-dependent antimicrobial activity of *H. illucens* extracts against the bacterial species *E. coli*, *M. luteus*, *P. fluorescens* and *B. subtilis*. As shown in Fig. 4, the strongest inhibition of Gram-negative bacteria was observed for the aqueous extracts of *H. illucens* larvae reared on the high-protein (brewer's grains) and cellulose diets, whereas inhibitory activities against Gram-positive bacteria were highest for larvae reared on chitin, cellulose, bacteria and plant oil. In contrast, chloroform extracts generally displayed the highest overall activities against all bacterial species when larvae were reared on the lignin, bacteria and plant oil diets (Fig. 4). Taken together, the growth inhibition assays showed that the diet during rearing had a significant impact on the antimicrobial activities of larval extracts.

4. Discussion

The black soldier fly *H. illucens* is a farmed insect used for the bioconversion of organic waste such as liquid manure (Cicková et al., 2015). The larvae can thrive on diverse substrates with remarkably variable nutritional components, and they are renowned for their ability to live in habitats characterized by extraordinary numbers of pathogens. An efficient and highly adaptable response to different nutritional resources as well as a strong and efficient immune system is therefore required to survive under these hazardous and unpredictable environmental conditions. Nutrition and immunity determine the complex processes through which organisms interact with their environment and adapt to variable conditions.

Accordingly, we investigated the repertoire of immunity-related genes in *H. illucens* and the effect of different types of diet on the immunity-related transcriptome. We identified more than 50 genes encoding putative AMPs, an extraordinary number which has been reported before in only one other insect, the invasive harlequin ladybird *Harmonia axyridis* (Vilcinskas et al., 2013). The evolutionary expansion of genes encoding AMPs can be considered as a preadaptation that allows *H. illucens* larvae to survive in pathogen-dense environments and tolerate food containing large numbers of bacteria. Similar expansions of AMP genes have been reported in rat-tailed maggots of the drone fly *Eristalis tenax,* which have adapted to survive in polluted aquatic habitats and liquid manure (Altincicek and Vilcinskas, 2007), and in maggots of the blow fly *Lucilia sericata*, which are known to sanitize wounds with secreted AMPs (Pöppel et al., 2015).

Impressively, we identified 26 genes encoding putative defensins. According to our knowledge this is the highest number reported from animals and only one has previously been purified, characterized and shown to display activity against Gram-positive bacteria including MRSA (Park et al., 2015).

We found that the nutritional components of the diet have a remarkable impact on the expression of immunity-related genes in *H. illucens* larvae. As reported in earlier studies with other insects (Knorr et al., 2015; Freitak et al., 2014), we found that a diet supplemented with a mixture of bacteria induced the expression of several AMPs in *H. illucens*. Another recent study confirmed that the direct injection of bacteria also triggers immune responses in *H. illucens*, characterized by enhanced activities against *E. coli* and *M. luteus* (Zdybicka-Barabas et al., 2017). A diverse spectrum of AMPs promotes synergistic and potentiating activities between members of different AMP families (Rahnamaeian et al., 2015). The growth inhibition assays showed that the larval diet has a significant impact on antimicrobial activity (Fig. 4). Furthermore, the observed growth inhibition was also dependent on the bacterial species and the solvent used for extraction. In particular, the highest increase in antibacterial activity compared to the standard diet was observed for chloroform extracts of larvae fed on specific dietary additives, such as protein and plant oil, whereas aqueous extracts were more potent for larvae fed on diets supplemented with high protein content (brewer's grains), chitin and cellulose (Fig. 4). Our results show that diet not only has a significant impact

control genes across samples. The map is based on log2-transformed RPKM values shown in the gradient heat map (blue represents weakly-expressed genes, and red represents strongly-expressed genes). Whole larval samples: Con = control diet, HBact = high bacterial load, Cell = cellulose, Chit = chitin, Lig = lignin, ProtR = high protein, POil = plant oil, Mix = mixed diet. Larval tissues: FMGut = foregut and midgut, HGut = hindgut, SalGl = salivary glands.

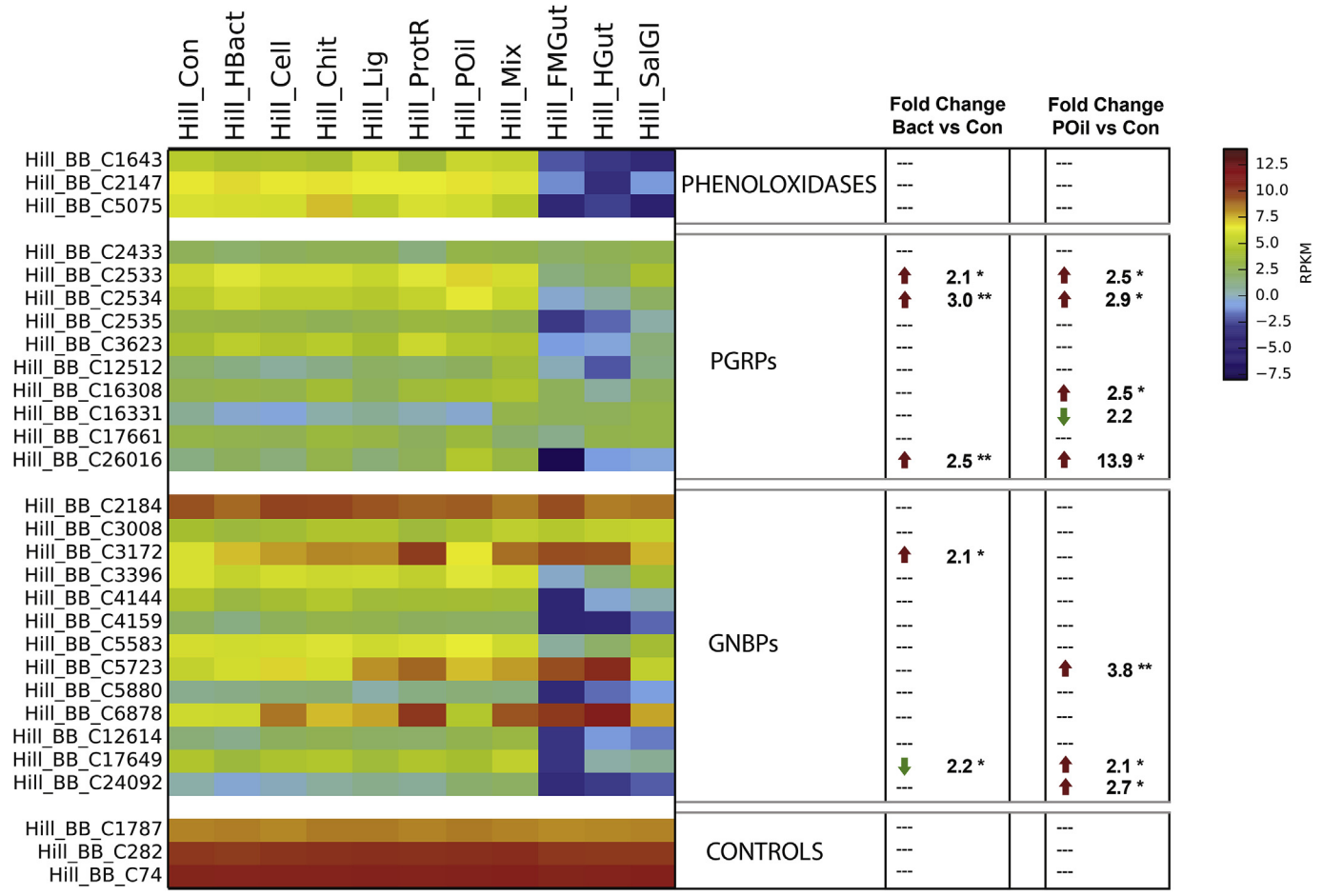

Fig. 2. Heat map showing the relative expression levels of phenoloxidases, PGRPs and GNBPs in *H. illucens* larvae in relation to different diets and tissues. Fold change gene expression differences among larvae exposed to high bacterial loads and plant oil-rich diets are compared to controls on the right. Red arrows indicate upregulation and green arrows indicate downregulation of the corresponding contigs. Significant differences (FDR-corrected p-values) are shown with an asterisk (* < 0.05, ** < 0.005). Genes of interest corresponding to the phenoloxidase, PGRP and GNBP families are grouped. The housekeeping genes *GAPDH*, *RPS4e* and *EIF5a* are used for normalization and are shown to confirm the uniform expression of these control genes across samples. The map is based on log2-transformed RPKM values shown in the gradient heat map (blue represents weakly-expressed genes, and red represents strongly-expressed genes). Whole larval samples: Con = control diet, HBact = high bacterial load, Cell = cellulose, Chit = chitin, Lig = lignin, ProtR = high protein, POil = plant oil, Mix = mixed diet. Larval tissues: FMGut = foregut and midgut, HGut = hindgut, SalGl = salivary glands.

on AMP gene expression but also on overall antimicrobial activities against a range of bacterial species. These observations are of economic importance given that the commercial mass rearing of insects aims to avoid the use of antibiotics (Grau et al., 2017). The "self-medication" of *H. illucens* would help to reduce the microbial load in larvae used to produce feed for livestock or aquaculture without additional costs.

Both constitutive and inducible immune responses are affected by host nutrition, which may in turn greatly affect the outcome of host infection and mortality (Ponton et al., 2011, 2013). However, although a limited dietary intake modulates the expression of immunity-related genes in some insects (Alaux et al., 2010; Brunner et al., 2014), these and most other studies have focused on dietary restrictions or changes of carbohydrate/protein ratios, rather than testing the effects of dietary ingredients (Lee et al., 2006; Simpson et al., 2015). Our experimental strategy, providing *H. illucens* larvae with a surplus of specific dietary ingredients on top of the standard diet, allowed us to identify immunity-related genes regulated at the transcriptional level in response to dietary components instead of dietary limitations. Unexpectedly, we found that feeding larvae with proteins or sunflower oil induced a stronger immune response than the addition of bacteria to the diet (Figs. 1 and 2). Our data also suggest that the effects of plant oils containing fatty acids and phytosterols on insects are more complex than previously envisaged. The immunomodulatory effects of phytosterols (such as sitosterol, which is abundant in sunflower oil) have been predominantly demonstrated in mammals, but are also proposed to play a role in insects (Xiangfeng et al., 2012).

In the burying beetle *N. vespilloides*, a comprehensive recent study has revealed the tightly regulated expression of an expanded spectrum of AMPs in functionally distinct compartments of the gut, along with distinct microbial communities in these compartments (Vogel et al., 2017). The authors postulated that the expanded and fine-tuned expression of AMPs in *N. vespilloides* supports the adaptation of both the flexible, environmentally modulated microbiota, and the host-adapted core microbiota, to facilitate the digestion of carcasses. This hypothesis is supported by the observation that burying beetles regulate the microbiome of carcasses and use it to transmit a core microbiota to their offspring (Shukla et al., 2017; Wang and Rozen, 2017). In analogy, we postulate that the expanded spectrum and diet-dependent expression of *H. illucens* AMPs is required to adapt the environmental microbiome (taken up with the diet) and the host-associated core microbiome to allow the digestion of flexible diets. Our current

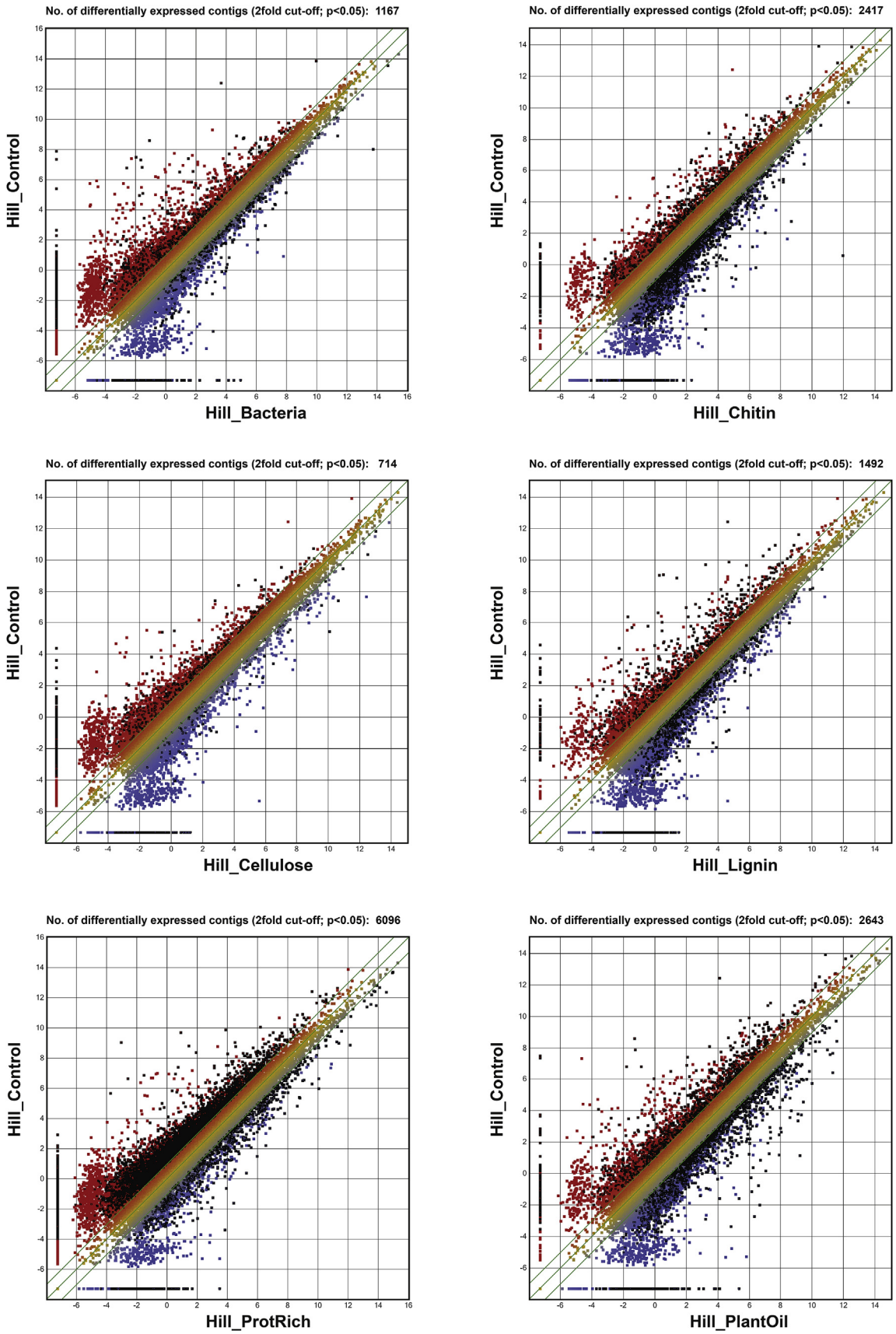

Fig. 3. Scatter plots of differentially expressed contigs in *H. illucens* larvae fed on different diets. The controls (y-axis) are compared to the different diets (x-axis). Contig expression ratios that are greater than two-fold and display a FDR-corrected p-value <0.05 are indicated with black dots. The total number of differentially expressed contigs is shown above each scatter plot.

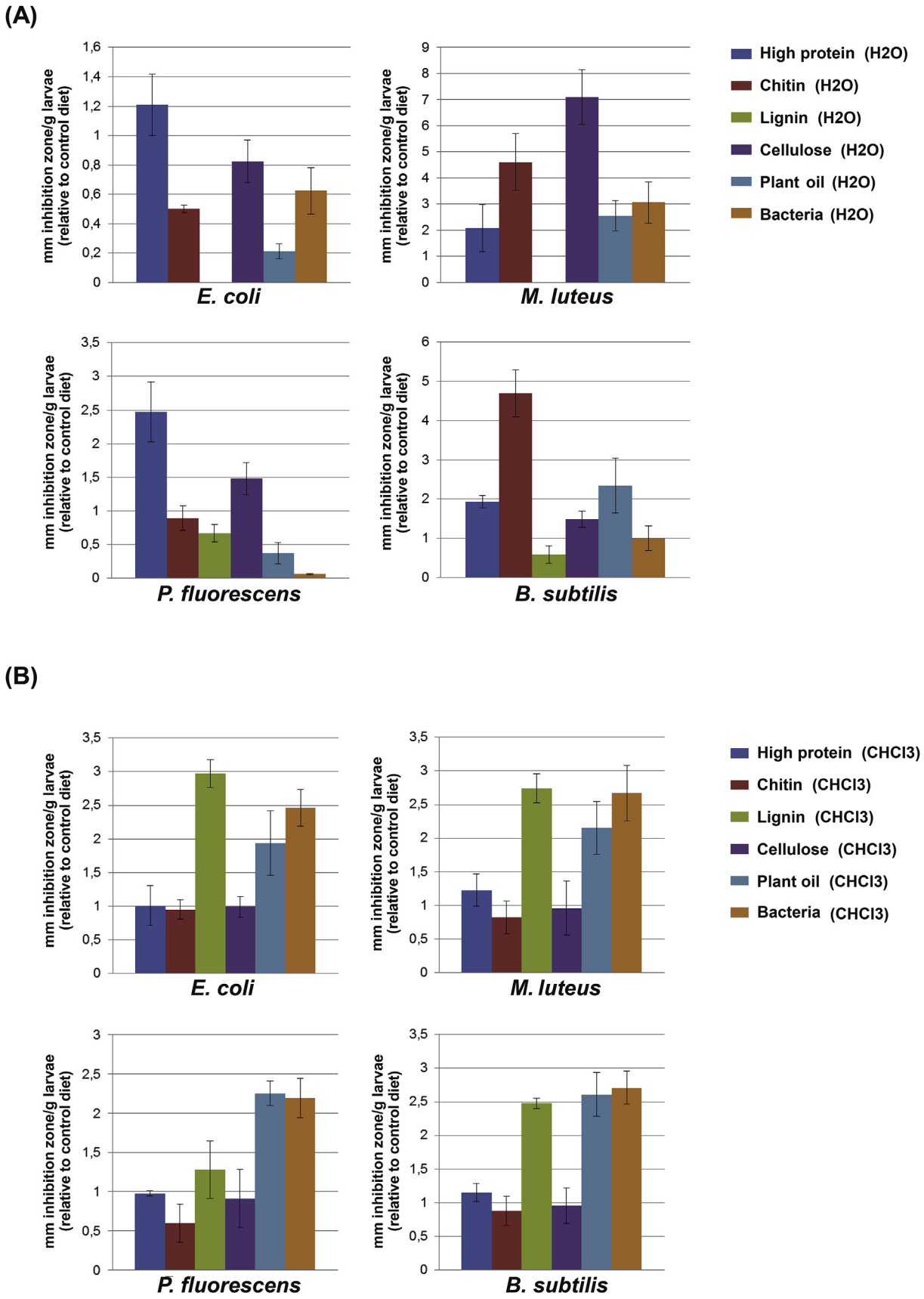

Fig. 4. Inhibition zone assays with *H. illucens* larval extracts feeding on different diets. Inhibition zones (mm per g larval starting material) are shown for four different bacteria. (A) Methanol-water extracts. (B) Chloroform extracts. The results are mean values ± s.e. Calculated from three independent experiments.

research therefore focuses on the characterization of diet-dependent changes of the gut microbiota in *H. illucens*. The diet-dependent expression of AMPs in *H. illucens* provides an intriguing example for the emerging field of nutritional immunology.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2017.09.008.

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