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RESEARCH ARTICLE

Microbiome symbionts and diet diversity incur costs on the immune system of insect larvae

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ABSTRACT

Communities of symbiotic microorganisms that colonize the gastrointestinal tract play an important role in food digestion and protection against opportunistic microbes. Diet diversity increases the number of symbionts in the intestines, a benefit that is considered to impose no cost for the host organism. However, less is known about the possible immunological investments that hosts have to make in order to control the infections caused by symbiont populations that increase because of diet diversity. Using taxonomical composition analysis of the *16S rRNA* V3 region, we show that enterococci are the dominating group of bacteria in the midgut of the larvae of the greater wax moth (*Galleria mellonella*). We found that the number of colony-forming units of enterococci and expressions of certain immunity-related antimicrobial peptide (AMP) genes such as *Gallerimycin*, *Gloverin*, *6-tox*, *Cecropin-D* and *Galiomicin* increased in response to a more diverse diet, which in turn decreased the encapsulation response of the larvae. Treatment with antibiotics significantly lowered the expression of all AMP genes. Diet and antibiotic treatment interaction did not affect the expression of *Gloverin* and *Galiomicin* AMP genes, but significantly influenced the expression of *Gallerimycin*, *6-tox* and *Cecropin-D*. Taken together, our results suggest that diet diversity influences microbiome diversity and AMP gene expression, ultimately affecting an organism's capacity to mount an immune response. Elevated basal levels of immunity-related genes (*Gloverin* and *Galiomicin*) might act as a prophylactic against opportunistic infections and as a mechanism that controls the gut symbionts. This would indicate that a diverse diet imposes higher immunity costs on organisms.

KEY WORDS: Antimicrobial peptides, Bacterial endosymbionts, Encapsulation response, Diet diversity, *Galleria mellonella*, Immunity

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INTRODUCTION

Pathogens are a constant threat to organisms (Schmid-Hempel, 2011), and so they need a surveillance system that recognizes and attacks the intruders. These may consist of viral, bacterial, fungal and parasitic infections or even the body's own non-cooperative cells, such as cancer. The innate immunity system is important because it starts acting immediately after a pathogen breaches the host's defense system (Janeway et al., 2005). The responses of the innate immunity system are more general than the more pathogen-specific responses of the adaptive immune system. The production of antimicrobial peptides (AMPs) is an early component of an innate immune response towards bacterial and fungal infections. AMPs are a group of antibiotics that impose a lethal effect against invading organisms by interfering with their basal biochemical functions (Zasloff, 2002; Brogden, 2005). Various AMPs modulate pathogen load and prevent the occurrence of an infection (Kaneko et al., 2007).

Immune function is expensive in terms of energy expenditure (e.g. Lochmiller and Deerenberg, 2000; Ardia et al., 2012), immunopathology (e.g. Råberg et al., 1998) and depletion of somatic resources (Adamo et al., 2008; Krams et al., 2012). This suggests that there are trade-offs in the allocation of resources between immune function and other components of fitness (e.g. Sheldon and Verhulst, 1996; González-Santoyo and Córdoba-Aguilar, 2012). Access to food and the nutritional quality of food are of particular importance in trade-offs between life history traits and nutrients. For example, the nutritional state of the host and the nutritional quality of its diet may have a profound effect on life history trade-offs and the ability to resist an infection (Alonso-Alvarez and Tella, 2001; Moret and Schmid-Hempel, 2000; Ponton et al., 2013; Povey et al., 2014). This suggests that food limitation and a low quality diet are likely to compromise the host's immune response (Smits et al., 2017). However, different levels of food limitation may produce different immune response patterns (e.g. Krams et al., 2014). For example, brief food deprivation may lead to an increase in the expression of AMP genes in the fruit fly *Drosophila melanogaster*, even in the absence of pathogens (Becker et al., 2010). This response is the opposite of what would be predicted if the immune system gradually declined as resources dwindle. Moreover, recent research shows that intracellular immune signaling pathways are interconnected with nutrient signaling pathways (e.g. in invertebrates, Becker et al., 2010; Adamo et al., 2016; in vertebrates, Odegaard and Chawla, 2013), providing an evolutionary explanation for the complex effects of food deprivation on immunity.

Epithelial cells of the gastrointestinal tract are covered with a large population of normally nonpathogenic bacteria. A fundamental part of digestion, these nonpathogenic bacteria are known as 'the microbiota'. Associations between the host and its microbiota are often mutualistic, which facilitates the establishment

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 4204-4212 doi:10.1242/jeb.169227

of persistent colonization of host organs and provides a variety of benefits to the hosts (Kau et al., 2011; Huang and Douglas, 2015; Masson et al., 2016). The microbiota competes with opportunistic microbes for nutrients and for attachment sites on epithelial surfaces by producing various antimicrobial substances. When the microbiota are affected by antibiotic treatment, pathogens may replace mutualistic microorganisms and cause diseases such as gastroenteritis, metabolic imbalance, inflammatory bowel disease and colorectal cancer (see Garrett et al., 2010). Moreover, the host's failure to regulate the survival and numbers of mutualistic microorganisms can allow normally nonpathogenic bacteria to grow excessively and also cause disease. A balance between bacterial growth and efficiency of nutrient processing in the gut and intestines is maintained by immunity mechanisms, such as generation of reactive oxygen species (De Block and Stoks, 2008; Jones et al., 2012) and AMPs (Login et al., 2011; Johnston and Rolff, 2015; Makarova et al., 2016). Overall, the composition of gut microbiomes is structured by (the host's) diet (Muegge et al., 2011). Some studies show that diet diversity correlates positively with microbiome diversity, leading the microbiome to be more adaptable to perturbations (reviewed in Heiman and Greenway, 2016, but see Bolnick et al., 2014). Previous studies on Lepidoptera (Broderick et al., 2004; Xiang et al., 2006; Pinto-Tomás et al., 2011; Gayatri Priya et al., 2012; Mason and Raffa, 2014) confirm the positive relationship between diet diversity and larval midgut community composition. The prediction arises that food limitations potentially decrease microbiome diversity. This may relieve the organism's investment in immune mechanisms of microbiota control in general and expression of AMP genes in particular, a factor commonly overlooked in eco-immunological research.

It has been demonstrated that dysbiosis caused by the oral delivery of antibiotics eliminates the midgut microbiome of the larvae of the greater wax moth [*Galleria mellonella* (Linnaeus 1758)] and decreases the transcriptional activation of AMPs (Mukherjee et al., 2013). In this study, we expected that AMP genes in the midgut of greater wax moth larvae would be less expressed when grown on simple food of low nutritional value than when grown on macronutrient- or energy-rich food. We also fed larvae with an antibiotic cocktail to test whether AMP gene expression during dysbiosis is similar to the transcriptional activation of AMPs of larvae raised on nutrient-poor food.

In *G. mellonella*, *Enterococci mundtii* (syn. *Streptococcus faecalis* Andrewes and Horder) is considered to be the dominating bacterium found in the midgut of the host (Jarosz, 1979; Johnston and Rolff, 2015). The dominance of just one microorganism can be explained by the antibacterial properties of their larval food, which consists of beeswax and honey, and the ability of *E. mundtii* to survive under these conditions. This microbe is a heritable nutrient-providing symbiont that is transmitted vertically – from mother to offspring (Chen et al., 2016) – in *G. mellonella* (Bucher, 1963; Johnston and Rolff, 2015). We analyzed the V3 region of 16S rDNA to determine the taxonomical composition of microorganisms in the midgut samples of the larvae of *G. mellonella*. Using conventional bacterial culturing, we investigated the numbers of enterococci in the midgut microbiota of the larvae grown either on diverse or simple food, and either force-fed with antibiotics or receiving no antibiotic treatment (control group). Food-related stress has been shown to affect the arms of the immune system via an antagonistic cross-regulation between different components of the immune system (Krams et al., 2012). To determine any possible costs of AMP gene upregulation in *G. mellonella*, we tested whether higher levels of AMP gene expression suppress encapsulation ability (Kangassalo et al., 2016; Krams et al., 2016; Kecko et al., 2017), an important defence mechanism against parasitic protozoans and metazoans, fungi, mites and parasitoids such as wasp eggs or larvae (Gillespie et al., 1997; Lavine and Strand, 2002; Krams et al., 2017).

MATERIALS AND METHODS

Insects and food quality

We studied a captive population of *G. mellonella* consisting of individuals collected from natural populations in Estonia in summer 2014. Moths were reared in 2.4 litre plastic boxes at 28±1°C in the dark in Sanyo MIR-253 incubators. To study the effects of diet diversity on the expression of AMP genes of *G. mellonella* larvae, we assigned them to groups differing in the macronutritional diversity and energetic value of food. Each larva was placed individually into a plastic container (50 ml) with a lid and wire-mesh to allow ventilation and to prevent it from escaping. The larvae were kept on contrasting diets between hatching and post-hatch day 25.

The 'diverse diet' group received only food of high nutritional value and diversity, provided *ad libitum*. The 'simple diet' group received only food of low nutritional value and diversity, provided *ad libitum*. The diverse diet consisted of a mix of equal proportions of honey, glycerol, beeswax, dried milk, wheat flour, dry yeast, distilled water and two servings of corn meal. Foods in both diets were autoclaved at 121°C for 15 min. Under natural conditions, increased diet diversity typically means that a higher diversity of opportunistic microorganisms is ingested by a host. This was not the case in our study, however, because the larvae received bacteria-free food. The amount of energy associated with this food was estimated as ca. 16.90 kJ g^{-1} by a combustion calorimeter C 2000 basic (IKA®-Werke GmbH & Co. KG, Germany). The simple diet consisted of natural beeswax with a 5% admixture of corn meal. Beeswax is a natural polymer produced by bees, and it is considered to have an extremely low nutritional value. However, we observed the ability of some wild progenitors of our study population to reproduce solely on beeswax. Thus, the larvae of the simple food group received a slightly better food than pure wax.

On post-hatch day 26, one half of the larvae in both food groups received peroral antibiotics to eliminate their microbiota. These individuals were treated with antibiotics and antifungal drugs, which was done twice within an 8-h interval. We force-fed each larva with 10 µl of antibiotic cocktail consisting of water-soluble forms of ampicillin (Sigma-Aldrich, St Louis, MO, USA; code: A5354, concentration 50 mg ml^{-1}; Gram-positive bacteria including *Streptococcus pneumonia*, *S. pyogenes*, *Staphylococcus aureus*, enterococci and Gram-negative bacteria including Enterobacteriaceae), erythromycin (Sigma-Aldrich) (code: 45703, concentration 1 mg ml^{-1}; Gram-positive bacteria: streptococci, staphylococci and Gram-negative bacteria and some fungi), gentamicin (Sigma-Aldrich; code: G-1397 code, concentration 50 mg ml^{-1}; Gram-negative bacteria including *Pseudomonas*, *Escherichia coli*, *Enterobacter aerogenes*, *Proteus* spp., *Serratia marcescens*, *Klebsiella pneumonia* and Gram-positive *Staphylococcus*), kanamycin (Sigma-Aldrich; code: K0254, concentration 50 mg ml^{-1}; Gram-negative bacteria including *E. coli*, *Proteus* spp., *S. marcescens*, *K. pneumonia*) and ketoconazole (Sigma-Aldrich) solution in dimethyl sulfoxide (code: K0600000, antifungal agent given in concentration of 1 µg $larva^{-1}$). The antibacterial cocktail has previously been documented to significantly decrease the expression of AMP genes and stress-related genes in *G. mellonella* (Mukherjee et al., 2013).

We extracted RNA from the larvae 24 h after they received the first dose of antibiotics. We also extracted RNA from the larvae

Journal of Experimental Biology (2017) 220, 4204-4212 doi:10.1242/jeb.169227

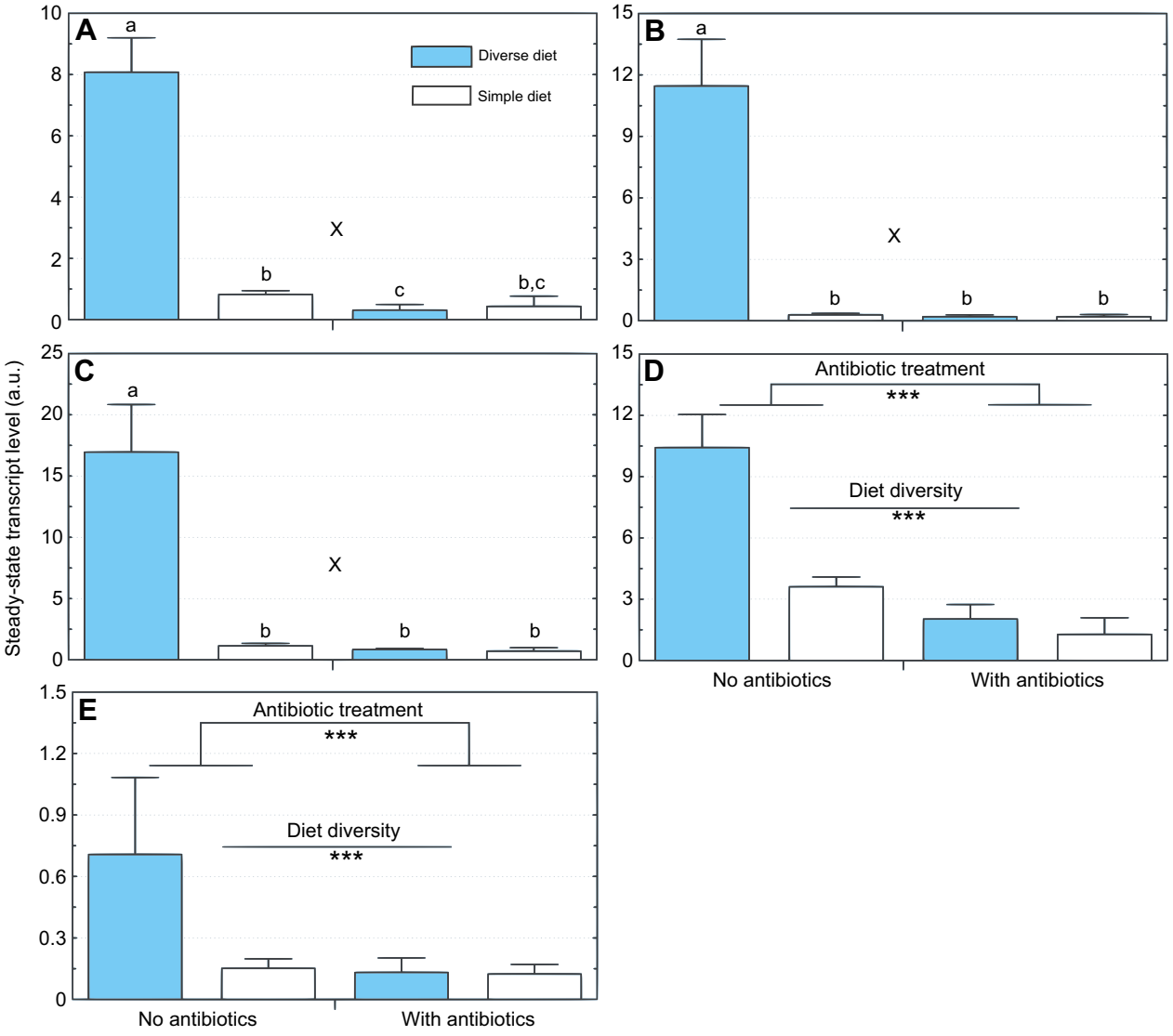

Fig. 1. The transcription levels of five AMP genes in the midgut of the greater wax moth larvae grown on a diverse and a simple diet with and without antibiotic treatment. (A) *6-tox*, (B) *Cecropin-D*, (C) *Gallerimycin*, (D) *Galiomicin* and (E) *Gloverin*. The transcription levels of the AMP genes were determined by a quantitative real-time RT-PCR analysis and are shown relative to the expression levels of the reference group in which the microbiome was eliminated. Results were normalized against the expression of the housekeeping *18S rRNA* and *EF1* genes and represent means of six independent determinations and standard deviations. Asterisks indicate significant main effects of diet and antibiotic treatment (***P<0.0001). X indicates a significant interaction between diet and treatment (P<0.05). Different lowercase letters denote significant differences by *post hoc* tests at P<0.05.

raised on diverse and simple foods that did not receive any antibiotics – these individuals were force-fed 10 µl of distilled water. In total, we had four experimental groups and one reference group of the greater wax moth. The experimental groups were as follows: (1) a control group raised on a diverse diet, (2) a control group raised on a simple diet, (3) an antibiotic treatment group raised on a diverse diet and (4) an antibiotic treatment group raised on a simple diet. The larvae of the reference group were raised on a simple diet. These larvae were force-fed 10 µl of distilled water every 3 h and received the antibiotics cocktail three times within a 12-h interval to eliminate their microbiome. RNA was extracted from these larvae 24 h after they received the first dose of antibiotics.

RNA extraction and quantitative real-time PCR of immunity-related gene expression

Collected larvae were chilled on ice for 15 min, surface sterilized with 70% ethanol and then dissected for collection of their midguts. We pooled midguts from 10 individual larvae for each group. RNA was obtained from five replicates of each of the four groups (200 larvae in total). The midguts were homogenized in 1 ml of Trizol reagent (Sigma-Aldrich), and RNA was extracted according to the manufacturer's recommendations. RNA integrity was confirmed by ethidium bromide gel staining, and quantities were determined spectrophotometrically.

Levels of steady-state transcripts were determined from cDNA samples by real-time quantitative RT-PCR using the $\Delta\Delta C_t$ protocol with the 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) and SYBR Green PCR mix (Qiagen, Venlo, The Netherlands). First-strand cDNA was synthesized using 5 µg of RNA, random hexamers and RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania) according to the manufacturer's protocols, and subsequently diluted with nuclease-free water (Fermentas) 10 to 100 times. Amplification mixtures (25 µl) contained 2 µl template cDNA, 2× SYBR Green Master Mix

Journal of Experimental Biology (2017) 220, 4204-4212 doi:10.1242/jeb.169227

buffer (12.5 µl) (Life Technologies, Carlsbad, CA, USA) and 2 µmol l^{-1} forward and reverse primer.

Five target genes were investigated, coding for AMPs: *Gloverin* (strong activity against gram-positive bacteria and weak activity against Gram-negative bacteria; forward primer: AGATGCACGGTCCTACAG, reverse primer: GATCGTAGGTGCCTTGTG), *Gallerimycin* (strong effect against filamentous fungi; forward primer: GAAGTCTACAGAATCACACGA, reverse primer: ATCGAAGACATTGACATCCA; Schuhmann et al., 2003), *6-tox* (an atypical defensin-derived immune-related peptide expressed in midgut against invading bacteria; forward primer: GACGAACTGCGAAGAATTATC, reverse primer: TGTCTGTCTTGAGTTGCATATTG; Lee et al., 2010), *Galiomicin* (strong antifungal effect and limited effect against bacteria; forward primer: GTGCGACGAATTACACCTC, reverse primer: TACTCGCACCAACAATTGAC) and *Cecropin-D* (strong activity against Gram-negative bacteria and fungi, weak activity against Gram-positive bacteria; forward primer: CTGCGCCATGTTCTTCA, reverse primer: TCGCATCTCTGATCCTCTG; de Melo et al., 2013).

Cycling conditions were 95°C for 5 min, followed by 42 cycles of: 95°C for 5 s, annealing for 10 s and 72°C for 20 s. An initial touchdown of 1°C per cycle from 65°C for the first five cycles resulted in optimal amplification for all loci. High-resolution melting analysis performed at the end of each run allowed each PCR to be checked for the presence of the expected product. We are confident that the efficiency was between 98.0% and 100% for all primer pairs. Every cDNA sample was normalized against two housekeeping genes: *18S rRNA* (AF286298; forward primer: CACATCCAAGGAAGGCAG, reverse primer: AGTGTACTCATTCCGATTACGA) and translation elongation factor 1-alpha (*EF1a*; AF423811; forward primer: AACCTCCTTACAGTGAATCC, reverse primer: ATGTTATCTCCGTGCCAG) (Vogel et al., 2011) using geNorm software (Vandesompele et al., 2002). The calculated gene expression stability coefficient *M* was applied to the assay results of the genes of interest. The primers were obtained from Metabion International AG (Planegg, Germany).

16S V3 rRNA gene amplification and Ion Proton™ semiconductor sequencing

The larvae used for gene sequencing received the same food as the diverse diet group, matching the conditions of *ad libitum* food availability in natural bee hives. DNA was extracted using Fast DNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's guidelines. The concentration of extracted DNA was measured using the Qubit® 2.0 Fluorometer High Sensitivity Assay (Life Technologies). The amount, average size and quality of the DNA were assessed using electrophoresis in 1.2% agarose gels. DNA was obtained from six laboratory populations and we used the midguts of six individual larvae per each sample (36 larvae in total).

The rRNA V3 region was amplified separately by reverse (Probio_Uni_R 5′-ATTACCGCGGCTGCT-3′) and forward (Probio_Uni_F 5′-CCTACGGGRSGCAGCAG-3′) primers (Milani et al., 2013). Both primers were tagged with 10–11 bp unique barcode labels along with the adapter sequence (5′-CCATCTCATCCCTGCGTGTCTCCGAC-3′). PCR amplification was performed using the GeneAmp® PCR System 9700 (Thermo Fisher Scientific, Waltham, MA, USA) with the PCR conditions used as follows: 98°C for 30 s, 35 cycles of 98°C for 10 s, 67°C for 15 s, 72°C for 15 s with the final extension at 72°C for 7 min. *16S rRNA* PCR products were then quantified, pooled and purified using the NucleoMag® NGS Clean-Up and Size Select kit (Macherey-Nagel, Düren, Germany). The quality and acquired amount of *16S rRNA* V3 amplicons was assessed by an Agilent High Sensitivity DNA kit on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA).

Prior to clonal amplification, each library was diluted to 8 pmol l^{-1} and pooled. Sample emulsion PCR, emulsion breaking and enrichment were performed using the Ion PI™ Hi-Q™ OT2 Kit (Life Technologies), following the manufacturer's instructions. The complete sample was loaded onto a PI™ chip v3 and sequenced on the Ion Proton™ Semiconductor Sequencer for 520 cycles employing the Ion PI™ Hi-Q™ Sequencing 200 Kit. Bidirectional sequencing was performed (i.e. sequence reads started from forward and reverse PCR primers), but reads were not paired. Each run was expected to produce approximately 240,000 reads. After the sequencing run was completed, the individual sequence reads were filtered by the Proton software to remove low quality sequences. Sequences matching the Proton 3′ adaptor were automatically trimmed. All Proton quality-approved, trimmed and filtered data were exported as bam files.

Sequencing data analysis was carried out using QIIME v.1.8.0 and UPARSE v.7.0.1001 pipeline to quality-filter and cluster 16S rRNA amplicon sequences (Pylro et al., 2014). Quality control retained sequences with the mean sequence quality score >20. Operational taxonomic units (OTUs) were built at 97% sequence identity with uclust (Edgar, 2010). Taxonomic assignment to the lowest possible rank was performed with RDP (Wang et al., 2007), using the Greengenes (DeSantis et al., 2006) (http://greengenes.lbl.gov) reference dataset (gg_otus-13_8 release). An alpha diversity measure – the Shannon diversity index – was calculated within the QIIME environment.

Conventional bacterial culturing

Midguts from six individual larvae were dissected and pooled for each of 20 replicates in four groups (480 larvae in total) and used for microbiological analysis. We homogenized 0.1 ml of the midgut samples with 1 ml of a sterile 0.1% peptone water solution using a laboratory paddle sampler for 3 min. Serial dilutions of the midgut homogenate were prepared with sterile peptone water and plated in duplicates on Bile Esculin Azide Agar (Sigma-Aldrich), a selective *Enterococcus* agar. Enterococci were further determined by the BBL Crystal Identification Systems Gram-positive ID kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) (e.g. Von Baum et al., 1998).

Immune assays

The strength of encapsulation response was measured in the control larvae raised on (1) the diverse diet (n=28) and (2) the simple diet (n=27). We also used *G. mellonella* larvae of the antibiotic treatment group raised on (3) the diverse diet (n=29) and (4) the simple diet (n=26). However, these were different larvae than those used for RNA extraction and conventional bacterial culturing.

The immune treatment of *G. mellonella* larvae on post-hatch day 25 consisted of an immune system challenge induced with a disinfected nylon monofilament implant (2 mm length, 0.18 mm diameter, knotted at one end) which was inserted between the third and fourth sternites of the cuticle of the larvae (Krams et al., 2014; Kecko et al., 2017) for 10 h at 28±0.5°C. The implant was removed after this treatment. The strength of the encapsulation response was measured as the darkness of the nylon filament insert after it had been dried. Insect immune systems respond to this challenge by attempting to encapsulate the implant in a coating of cellular materials and chemical deposits as if the insert were a foreign body

Journal of Experimental Biology (2017) 220, 4204-4212 doi:10.1242/jeb.169227

or parasite (e.g. Rantala et al., 2000). The stronger the immune response to the insert, the darker the encapsulation and the higher its grayscale value (Yourth et al., 2001; Krams et al., 2011, 2017; Daukšte et al., 2012) owing to phenoloxidase enzyme production activated by the immune response, resulting in melanization of the capsule (Ratcliffe et al., 1985). In the greater wax moth, the encapsulation response has been confirmed to be associated with parasitoid (Kryukova et al., 2011), fungal (Dubovskiy et al., 2011, 2013) and bacterial infections (Grizanova et al., 2014). The intensity of melanization was assessed from photographs of the inserts taken from three directions under constant light conditions using a Zeiss Lumar V12 stereo microscope (Carl Zeiss, Jena, Germany). Digital images were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/; Abramoff et al., 2004). Before the insertion of the monofilament implant, we calibrated the reflectance of the implant to zero level.

Data analysis

As the Brown–Forsythe test showed a violation of the homogeneity of variance of gene expression levels and encapsulation rates, we used generalized linear models (GLM) with gamma error structure and an inverse link function to compare gene expression levels and encapsulation rates. Diet, treatment and their interaction were used as independent variables. If an interaction showed a significant effect, the Games–Howell *post hoc* test was used to account for unequal variance. The analysis was performed using R 3.3.2 (R Core Team, 2016) and the packages onewaytests (Dag et al., 2016) and userfriendlyscience (Peters, 2016). We compared the number of enterococci colony forming units (CFU) in diverse and simple diet groups (without antibiotic treatment) using a *t*-test. Data are expressed as means±s.d. where applicable.

RESULTS

The GLM analysis showed that expression of all the AMP genes was significantly higher in the diverse diet group than in the simple diet group [*Gallerimycin*: Rao–Scott modified likelihood ratio test (LR $Chisq_1$)=235.709, $P<0.0001$; *6-tox*: LR $Chisq_1$=40.384, $P<0.0001$; *Galiomicin*: LR $Chisq_1$=22.491, $P<0.0001$; *Cecropin-D*: LR $Chisq_1$=171.380, $P<0.0001$; *Gloverin*: LR $Chisq_1$=23.858, $P<0.0001$]. Treatment with antibiotics was associated with significantly lower expression of all AMP genes compared with groups without antibiotic treatment (*6-tox*: LR $Chisq_1$=77.948, $P<0.0001$; *Cecropin-D*: LR $Chisq_1$=201.231, $P<0.0001$; *Gallerimycin*: LR $Chisq_1$=288.633, $P<0.0001$; *Galiomicin*: LR $Chisq_1$=61.111, $P<0.0001$; *Gloverin*: LR $Chisq_1$=29.028, $P<0.0001$). The diet and treatment interaction showed a significant effect on expression of *Gallerimycin* (LR $Chisq_1$=7.322, $P=0.0068$), *6-tox* (LR $Chisq_1$=5.068, $P=0.0244$) and *Cecropin-D* (LR $Chisq_1$=6.983, $P=0.0082$), whereas *Gloverin* (LR $Chisq_1$=3.260, $P=0.071$) and *Galiomicin* (LR $Chisq_1$=0.438, $P=0.5079$) gene expression did not differ significantly between interaction groups, suggesting that diet alone has the potential to increase the expression of these two genes.

Post hoc tests revealed that the AMP genes were expressed at significantly higher levels in the diverse diet group than in the simple diet group (means±s.d.) when the larvae of *G. mellonella* were not treated with antibiotics (*Gallerymicin*: diverse=16.96±3.88 versus simple=1.15±0.2, $P=0.003$; *6-tox*: diverse=8.08±1.12 versus simple=0.82±0.05, $P<0.001$; *Cecropin-D*: diverse=11.46±2.27 versus simple=0.30±0.07, $P=0.001$; Fig. 1). However, we did not find any significant differences in AMP gene expression between the diverse and simple diet groups under antibiotics treatment (all $P>0.05$; Fig. 1A–C). In general, the AMP genes were similarly expressed between the simple diet without antibiotics treatment group and the diverse diet and simple diet groups with antibiotics treatment ($P>0.05$). The exception was *6-tox*, which was expressed significantly more in the simple diet without antibiotics group than in the diverse diet with antibiotics group ($P=0.006$; Fig. 1). Expression of *Cecropin-D*, *6-tox* and *Gallerymicin* genes in the diverse diet without antibiotics group was significantly higher than that in the diverse and simple diet groups with antibiotics (all $P<0.005$; Fig. 1).

Sequencing the midgut samples of *G. mellonella* by the Ion Proton™ semiconductor sequencer resulted in 248,073 sequences per sample, but the number of sequences retained after the quality control was 177,742. Taxonomical composition analysis of the *16S rRNA* V3 region revealed that the most prevalent genus in the microbial community associated with the midgut of *G. mellonella* larvae was *Enterococcus* (ca. 80% of the sequences), while the relative abundance of the family Enterococcaceae accounted for 87% of the microbiome.

The highest number of CFU of enterococci was found in the diverse diet without antibiotics group ($7.6\times10^6\pm14.70\times10^6$ CFU ml^{-1}), while the number of enterococci CFU was significantly lower in the simple diet group ($0.8\times10^3\pm1.5\times10^3$ CFU ml^{-1}; *t*-test: $t_{38}=2.31$, $P=0.027$). We did not find any bacteria in the midgut of the individuals in the antibiotic treatment groups grown on diverse and simple diets (Fig. 2).

The encapsulation response was affected by diet (GLM, LR $Chisq_1$=371.42, $P<0.0001$), treatment (LR $Chisq_1$=24.78, $P<0.0001$) and interaction between diet and treatment (LR $Chisq_1$=7.32, $P=0.007$). The Games–Howell *post hoc* test showed that there are significant differences (all $P<0.01$; Fig. 3) in encapsulation response between the simple diet without antibiotics treatment group (29.04±2.47), the simple diet with antibiotics treatment group (35.88±4.26), the diverse diet without antibiotics group (19.50±3.17) and the diverse diet with antibiotics group (19.76±3.16). The only exception occurred between the diverse diet without antibiotics and the diverse diet with antibiotics treatment groups, which did not differ significantly ($P>0.05$; Fig. 3).

DISCUSSION

Alpha diversity measurements of the relative abundance of bacteria have shown that enterococci are the most common group of microorganisms in the midgut microbiome of *G. mellonella* (Jarosz, 1979; Johnston and Rolff, 2015). Our results confirm those findings and support other findings on how the elimination of nutrients

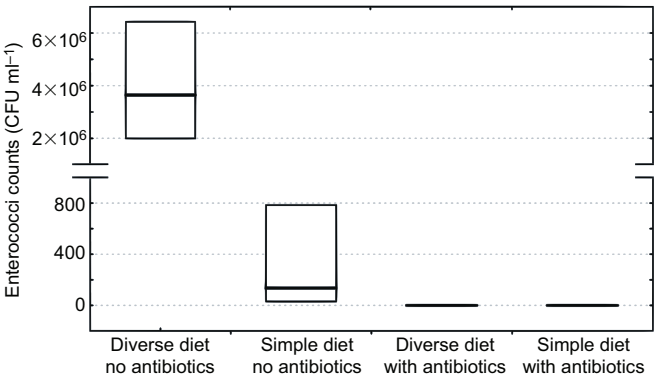

Fig. 2. Enterococci counts (CFU ml^{-1}) in the midgut samples of *Galleria mellonella* in four experimental treatments. Thick lines represent medians, while the boxes show 25th–75th percentiles.

Journal of Experimental Biology (2017) 220, 4204-4212 doi:10.1242/jeb.169227

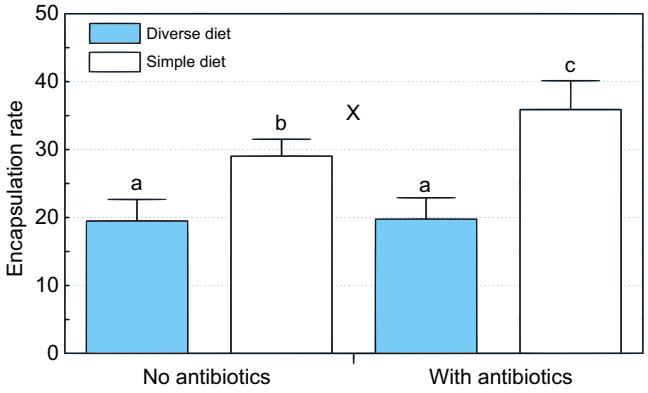

Fig. 3. Encapsulation rate (mean±s.d.) of the greater wax moth larvae grown on the 'diverse diet' and the 'simple diet' with and without antibiotic treatment. X indicates a significant interaction between diet diversity and treatment (P<0.01). Different lowercase letters denote significant differences by *post hoc* tests at P<0.01.

reduces investments in the immune system (Alonso-Alvarez and Tella, 2001; Krams et al., 2014), while also reducing symbiont numbers and microbiota diversity (David et al., 2014; Carmody et al., 2015; Sonnenburg et al., 2016). This highlights the importance of food diversity and quality in shaping intestinal microbiota, which may have profound effects on organismal growth trajectories and the evolution of reproductive trade-offs (Lazzaro and Rolff, 2011).

The results of this study show that when force-fed with antibiotics at larval stage, *G. mellonella* developed basal expression levels of immunity-related AMP genes. We suggest that these levels reflect 'surveillance' activity of the immune system in the midgut in the absence of symbionts. Interestingly, the basal expression of *Cecropin-D*, *Gallerimycin* and *6-tox* of larvae force-fed with antibiotics did not differ from expression of those genes under conditions when the larvae received a simple diet without antibiotics. This shows that investment in the production of AMPs remains the same between conditions of microbial dysbiosis and the diet consisting of simple/low-energy food despite the difference in symbiont numbers in the midgut. This result suggests the leading role of diet played in the expression of AMPs. In contrast, the AMP genes *6-tox*, *Cecropin-D*, *Galiomicin*, *Gallerimycin* and *Gloverin* were all significantly upregulated in the group of larvae grown on a diverse diet−those harbouring the highest number of enterococci symbionts. Hence, the elevated gene expression was correlated with the increased diet diversity and the number of enterococci symbionts as revealed by conventional bacterial culturing. This positive association between the increased number of symbionts and elevated immune-related gene expression shows that diet quality and/or diversity may result in a higher number of symbionts involved in the digestion of nutrients, and this presumably requires more control over symbionts by means of AMP proteins.

The gastrointestinal microbiome is considered to be more diverse in the intestines of healthy organisms, while a loss in species diversity is a common finding in several disease states (for a review, see Heiman and Greenway, 2016). However, symbionts may become harmful if they grow and reproduce uncontrollably. Symbionts may therefore consume more nutrients than would be normally expected to maintain symbiotic relationships, causing bacterial overgrowth in the intestines and/or midgut if not properly controlled by the immune system of the host (Tamboli et al., 2004; Erdogan and Rao, 2015; Fujimori, 2015; Moos et al., 2016). For example, insects are known to use protective bacterial symbionts to provide additional defense against their predators and parasitoids (Oliver et al., 2010; Polin et al., 2014). However, these symbioses have been shown to induce costs. For example, while bacterial symbionts provide the pea aphid (*Acyrthosiphon pisum*) with strong protection against parasitoids, this symbiosis becomes costly in the presence of predatory ladybirds because aphids infected by protective symbionts express less defensive anti-predator responses (Polin et al., 2014). Luo et al. (2017) have recently investigated the ecological effects of a non-essential secondary endosymbiont *Regiella insecticola* on different clones of the English grain aphid (*Sitobion avenae*) based on the life table data. They showed that *R. insecticola* can decelerate the normal development of the hosts. Similarly, our results suggest that high numbers of symbiotic enterococci bacteria (Johnston and Rolff, 2015) are involved in the elevation of costly expression of immune-related genes (for example, *Gloverin*), an outcome that may be further influenced by the presence of other microorganisms undetected or not evaluated in our study. The 'surveillance' of symbionts and the activation and production of AMPs in cases when the symbionts become too abundant are important functions. These functions might incur costs, as the larvae would need to divert resources and energy away from growth and other organismal needs in order to carry out those functions. A fine-grained analysis of these potential trade-offs requires a more detailed experimental approach in future studies.

Our findings are indirectly aligned with previous studies concerning the role of host immunity in shaping microbiota. The gut microbiome often represents a complex and highly dynamic biological system (e.g. Lanan et al., 2016). Symbiont communities within the gut lumen may be unstable owing to the constant influx of food particles (Blum et al., 2013) and invasions of other non-symbiotic food-associated bacteria (Jones et al., 2013; Cariveau et al., 2014). Thus, the number of bacteria and the diversity of insect microbiome can be explained by bacterial competition in the midgut (Greenberg and Klowden, 1972). Another hypothesis suggests that the immune system of the host plays an important role in the formation and maintenance of bacterial assemblages in the midgut of insects (Russell and Dunn, 1996; Caccia et al., 2016). Other recent work shows that host and symbiont communities cooperatively interact to maintain the midgut microbiota in a symbiotic balance (Johnston and Rolff, 2015). Our results suggest that all of the above ideas are worth testing by manipulating the levels of food resources available to the host, which we found to be an important agent affecting symbiont numbers and the strength of AMP gene expression.

Importantly, our results suggest that food diversity has the potential to influence the immune system, as shown by the elevated expression of *Gloverin* (responsible for suppressing Gram-positive bacteria such as enterococci) and *Galiomicin* AMP genes. This may be adaptive under natural conditions because the larvae of *G. mellonella* often invade only hives with low honey bee populations or hives where bees have already died from a disease (de Barjac and Thompson, 1970). However, the hives are not kept as clean as those maintained by healthy bee colonies. This suggests that the food of *G. mellonella* is often contaminated by various bacteria and fungi. Thus, a diverse diet in nature might indicate a higher probability of acquiring opportunistic infections, while an elevated expression of *Gloverin* and *Galiomicin* AMP genes may be considered to be a prophylactic response to pathogens (Barnes and Siva-Jothy, 2000).

It has recently been shown that a high-energy and/or diverse diet decreases the strength of the encapsulation response, which indicates that food quality is the primary determinant of encapsulation response in the larvae of *G. mellonella* (Krams et al., 2014). Because the larvae grow the fastest when raised on a high-quality diet, it was suggested that longer larval development on low-quality diet requires enhanced encapsulation reactions (Krams et al., 2014). The present study, however, reveals one more mechanism that may link the diverse and/or high-quality diet with weaker encapsulation responses in *G. mellonella*. We show that the elevated expression of all AMP genes is associated with the diverse diet and that this result was independent of the antibiotics treatment. Interestingly, the encapsulation response was stronger in the simple diet with antibiotics treatment group, which indicates that the encapsulation response is also dependent on the presence of bacteria, but only when grown on the simple diet. This shows that the diverse diet itself provides a signal of possible bacterial contamination which decreases the encapsulation rate and elevates the AMP protection because the latter is more important in fighting opportunistic infections. However, what needs to be investigated in the future is whether the relationship between the production of AMP peptides and the strength of the encapsulation response is a result of stress-induced suppression of the humoral immune response manifesting through an antagonistic cross-regulation between different components of the immune system (Sapolsky et al., 2000; Dhabhar, 2009) or whether the encapsulation response and AMP gene expression are a part of the same protective mechanism. For example, Nagai et al. (2001) found that tachyplesin, a major chitin-binding AMP of the horseshoe crab (*Tachypleus tridentatus*), induces the synthesis of phenoloxidase, which is a crucial part of the host defense system including the synthesis of melanin and facilitation of encapsulation response. Phenoloxidase, a copper-containing enzyme, is known to be responsible for initiating the biosynthesis of melanin, which is used during the encapsulation response (e.g. Hoffmann et al., 1999).

In conclusion, although symbiotic interactions are supposed to be mutually beneficial to the host and its symbionts, this study suggests that high numbers of midgut symbionts may induce ecological costs to their insect hosts. This conclusion is based on the finding that a diverse diet (and, consequently, a higher number of microbiome symbionts) activates the part of the immune system of *G. mellonella* larvae that is responsible for the production of AMPs. More specifically, we found that the number of CFU of enterococci and expression of *Gallerimycin*, *Gloverin*, *6-tox*, *Cecropin-D* and *Galiomicin* increased in response to a more diverse diet. However, quantifying and revealing the exact mechanisms of these costs as a part of ecological trade-offs remain to be done in future research. This task will require studies to disentangle the possible effects caused by a diverse diet (Huang and Douglas, 2015). This can be accomplished by eliciting the prophylactic activation of the immune system and analyzing the relationship between diet diversity and the number of symbionts, which seemingly needs to be controlled by the host. Finally, food availability is an important growth determinant not only for the host but also for its symbionts. Because the availability of food resources has the potential to affect symbiont numbers and microbiota species composition, these effects need to be taken into account while designing and performing future experiments. This type of work is crucial in order to increase our understanding of the ecological effects that food resources have on immunity, growth and reproduction trade-offs under the immense complexity of food webs and their dynamic interactions with the microbiota.

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Competing interests
The authors declare no competing or financial interests.

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