

RESEARCH ARTICLE

Pollen reverses decreased lifespan, altered nutritional metabolism and suppressed immunity in honey bees (*Apis mellifera*) treated with antibiotics

Jianghong Li^{1,2,*}, Matthew C. Heerman^{1,*}, Jay D. Evans¹, Robyn Rose³, Wenfeng Li¹, Cristina Rodríguez-García¹, Gloria DeGrandi-Hoffman⁴, Yazhou Zhao^{1,5}, Shaokang Huang², Zhiguo Li², Michele Hamilton¹ and Yanping Chen^{1,‡}

ABSTRACT

Nutrition is involved in regulating multiple aspects of honey bee biology such as caste, immunity, lifespan, growth and behavioral development. Deformed wing virus (DWV) is a major pathogenic factor which threatens honey bee populations, and its replication is regulated by the nutrition status and immune response of honey bees. The alimentary canal of the honey bee is home to a diverse microbial community that provides essential nutrients and serves to bolster immune responses. However, to what extent gut bacteria affect honey bee nutrition metabolism and immunity with respect to DWV has not been investigated fully. In this study, newly emerged worker bees were subjected to four diets that contained (1) pollen, (2) pollen and antibiotics, (3) neither pollen nor antibiotics or (4) antibiotics alone. The expression level of two nutrition genes *target of rapamycin (tor)* and *insulin like peptide (ilp1)*, one nutritional marker gene *vitellogenin (vg)*, five *major royal jelly protein* genes (*mrjp1–5*), one antimicrobial peptide regulating gene *relish (rel)*, and DWV virus titer and its replication intermediate, negative RNA strand, were determined by qRT-PCR from the honey bees at 7 days post-antibiotic treatment. Additionally, honey bee head mass and survival rate were measured. We observed that antibiotics decreased the expression of *tor* and *rel*, and increased DWV titer and its replication activity. Expression of *ilp1*, *mrjp1–5* and *vg*, and honey bee head mass were also reduced compared with bees on a pollen diet. Antibiotics also caused a significant drop in survivorship, which could be rescued by addition of pollen to the diet. Of importance, pollen could partially rescue the loss of *vg* and *mrjp2* while also increasing the head mass of antibiotic-treated bees. Our results illuminate the roles of bacteria in honey bee nutrition, metabolism and immunity, which confer the ability to inhibit virus replication, extend honey bee lifespan and improve overall health.

KEY WORDS: Gut bacteria, Nutrition metabolism, Immunity, Deformed wing virus, DWV

¹USDA-ARS Bee Research Laboratory, Building 306, BARC-East, Beltsville, MD 20705, USA. ²College of Bee Science, Fujian Agriculture and Forestry University, Fuzhou 350002, China. ³USDA APHIS, Plant Protection and Quarantine, 4700 River Rd, Riverdale, MD 20737, USA. ⁴USDA-ARS Carl Hayden Bee Research Center, 2000 East Allen Road, Tucson, AZ 85719, USA. ⁵Institute of Apicultural Research, Chinese Academy of Agriculture Sciences, Beijing 100081, China.

*These authors contributed equally to this work

‡Author for correspondence (judy.chen@ars.usda.gov)

ORCID C.R.-G., 0000-0002-0257-2745; Y.C., 0000-0002-5224-1100

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INTRODUCTION

Honey bees, as the most economically important pollinator, are indispensable for maintaining global ecological stability and agricultural production. However, the colony losses worldwide in the past decade threaten agricultural production and food supply (Garibaldi et al., 2011). Among the many threats affecting bee health, viruses are considered a key factor contributing to honey bee losses (Chen and Siede, 2007; Cox-Foster et al., 2007; Francis et al., 2013; Highfield et al., 2009; Ratnieks and Carreck, 2010). So far, more than 30 viruses have been reported to infect honey bees worldwide (Galbraith et al., 2018; Tantillo et al., 2015). Among them, deformed wing virus (DWV) is the most prevalent virus, persisting in all stages of honey bee development and in most apiaries in the world. Typically, DWV infections exist in a latent (covert) state and become actively pathogenic (overt) in the presence of stressors such as high infestation by the parasitic mite *Varroa destructor*, poor nutrition and lax beekeeping management practices. In these situations, DWV undergoes abundant replication, leading to colony losses (de Miranda and Genersch, 2010; Genersch and Aubert, 2010; Tantillo et al., 2015). DWV amount is also used as a metric to predict colony strength, and is taken as a negative marker of honey bee fitness (Budge et al., 2015).

Generally, the availability and quality of food fundamentally determine the distribution and scale of bee populations (Plascencia and Philpott, 2017; Smart et al., 2016). Previous reports have demonstrated that climate change and/or human activity may affect food availability and diversity, which is partially responsible for honey bee colony health (Donkersley et al., 2014; Morimoto et al., 2011; Ziska et al., 2016). Nutrition affects a variety of phenomena associated with honey bee biology and development. The quantity and duration of larval ingestion of royal jelly determines their future caste development as queen versus worker (Mutti et al., 2011; Patel et al., 2007; Wang et al., 2013). It also guides honey bees in the process of behavioral development (Ament et al., 2008, 2010; Toth et al., 2005). The three castes of honey bee – worker, queen and drone – have different brain development which is also tightly linked to nutrition (Moda et al., 2013). Overwintering worker bees normally have a larger portion of their body dedicated to the storage of nutrients than do bees from other seasons (Mattila and Otis, 2006; Mattila and Otis, 2007). Queens and egg-laying workers that develop within queenless colonies also have a greater need for nutrient storage and active metabolism (Hoover et al., 2006; Kucharski et al., 2008; Peso et al., 2016). Nutrient quality and quantity also affects host immunity through direct and indirect effects mediated by the host's microbiota and pathogen populations (Ponton et al., 2013). Many studies have demonstrated the role of nutritionally linked immunity in countering viruses and other

pathogens such as the intracellular microsporidian parasite *Nosema* (Basualdo et al., 2014; DeGrandi-Hoffman and Chen, 2015; DeGrandi-Hoffman et al., 2010; Glavinic et al., 2017; Xu et al., 2013; Zheng et al., 2014).

Insulin signaling and target of rapamycin (TOR) pathways are responsible for nutrient sensing and metabolism in honey bees (Ament et al., 2008; Ament et al., 2010; Nelson et al., 2007; Scofield and Mattila, 2015; Toth et al., 2005; Wheeler et al., 2014). Pollen is the primary protein source for honey bee growth and development, and has a fundamental effect on honey bee nutrient metabolism (Basualdo et al., 2014; Zheng et al., 2014). Vitellogenin (Vg), a glycoprotein secreted into the hemolymph by the fat body, governs a variety of physiological aspects including development, behavior, life span and immunity (Amdam et al., 2004; Corona et al., 2007; Münch et al., 2008; Nelson et al., 2007; Peso et al., 2016; Salmela et al., 2015), and is considered to be a general marker for honey bee health (Amdam et al., 2003; Dainat et al., 2012). Secretions of major royal jelly proteins from the hypopharyngeal glands (HPG) of nurse bees are fed to immature larvae and queens and are positively correlated with successful colony development (Wegener et al., 2009).

The honey bee midgut houses many microbes spread over a relatively limited number (nine) of bacterial phyla (Kwong and Moran, 2016; Martinson et al., 2012; Powell et al., 2014; Sabree et al., 2012). In particular, carbohydrate metabolism of gut bacteria is well adapted to the high sugar content of honey, fulfilling honey bee energy requirements (Kwong and Moran, 2015). Some strains of gut bacteria such as *Gilliamella apicola* are capable of degrading the cell walls surrounding pollen particles, allowing further digestion (Engel et al., 2012; Saraiva et al., 2015). Gut bacteria also promote the production of antimicrobial peptides (AMPs), which provide added immunity against parasites and pathogens (Butler et al., 2013; Evans and Armstrong, 2006; Glittenberg et al., 2011; Hooper et al., 2012; Kwong et al., 2017; Raymann et al., 2017; Vásquez et al., 2012; Zheng et al., 2017), thereby extending honey bee life expectancy (Raymann et al., 2017). Gut bacteria colonize and establish their population 3–5 days post-eclosion in honey bees (Hroncova et al., 2015; Martinson et al., 2012; Powell et al., 2014). This initial microbial community is crucial for an active metabolism capable of producing and secreting royal jelly associated with nursing bees 5–12 days post-eclosion (Ohashi et al., 1997). Previous work demonstrated that the microbial flora varies significantly across worker, queen and drone bees, likely owing to their different energy requirements (Kapheim et al., 2015). Taken together, these studies point to an intimate relationship involving microbial communities, nutrient metabolism and honey bee immunity.

In this study, we determined the effect of gut bacteria disruption by antibiotics on nutrient metabolism, immunity, quantity and replicative state of DWV, and lifespan of honey bees. The results demonstrate gut bacteria disruption decreases honey bee metabolic activity, immunity and lifespan while increasing DWV replication. Our results and observations provide a deeper understanding of the interconnections among gut bacteria, honey bee nutrition and immunity.

MATERIALS AND METHODS

Ethics statement

No specific permits were required for the studies described. Observations were made in the United States Department of Agriculture (USDA)-Agricultural Research Service (ARS) Bee Research Laboratory apiaries, Beltsville, MD, USA. The apiaries are the property of the USDA-ARS and are not privately owned or

protected in any way. Studies involved the European honey bee (*Apis mellifera* Linnaeus 1758) which is neither an endangered nor a protected species.

Honey bees used in this study

Experimental honey bees were obtained from healthy bee colonies maintained at the USDA-ARS Bee Research Laboratory. Brood frames with emerging bees and food stores were collected and individually placed in a mesh-walled cage and incubated in an insect growth chamber at 34°C and 55% relative humidity (RH) as described previously (Evans et al., 2009). Newly emerged bees were left roaming on the frame for 2 days (48 h) so they could be naturally inoculated by residual gut symbionts on the frame surface.

Pollen and antibiotic treatments

After confirmation of the acquisition and abundance of bacterial phlotypes, the 48 h old bees were collected and divided into four groups. Bees were subjected to the following four treatments: group I bees were fed with pollen collected from pollen traps that had been placed on field colonies and a 50% sugar solution (w/v) as a positive control; group II bees were fed pollen and a 50× dilution of penicillin (10,000 units ml⁻¹)–streptomycin (10,000 µg ml⁻¹) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 50% sugar solution (w/v); group III bees were fed with a 50% sugar solution containing neither pollen nor antibiotics; and group IV bees were fed a 50× dilution of penicillin–streptomycin (Thermo Fisher Scientific, Inc.) in a 50% sugar solution (w/v) and no pollen. Each group consisted of six cages with 40 bees per cage. For each group, three cages were used for determining the survival rate of bees in each treatment, and the remaining three cages were used for gene expression analysis. Honey bees were reared in an insect incubator at 32.5°C and >80% RH as previously reported (Evans et al., 2009). Cages were checked daily to adjust sugar solution and pollen supply, and dead bees were removed and counted for analysis of survival rates of the respective groups. Four honey bees were collected per cage at 7 days post-treatment. The heads were removed and weighed on an analytical balance (Mettler Toledo, Columbus, OH, USA). Head mass was used as an indicator of hypopharyngeal gland development (Hrassnigg and Crailsheim, 1998). The bees were then frozen at –80°C for RNA extraction and downstream analysis.

DNA and RNA extraction

Honey bee samples were removed from the –80°C freezer and immediately ground with a plastic pestle in a 1.5 ml Eppendorf tube individually. For DNA isolation, ground bees were homogenized in 1 ml DNAzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and the DNA was precipitated with ethanol, washed and dissolved in 100 µl nuclease-free water. For RNA isolation, 1 ml Trizol (Invitrogen) was added to the tube following the manufacturer's instructions. The extracted RNA was dissolved in 100 µl nuclease-free water with 2 µl ribonuclease inhibitor (Invitrogen). The quantity and purity of DNA and RNA were measured using a NanoDrop 8000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA samples were stored at –20°C and RNA samples were stored at –80°C until used.

Bacterial phylotype analysis using quantitative PCR (qPCR)

The acquisition and abundance of bacterial phlotypes were confirmed by sampling 12 newly emerged bees and 12 adult (48 h old) bees for qPCR analysis following the method described previously (Li et al., 2017). The abundance of gut bacteria species

clusters (*Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, *Bifidobacterium asteroides*), which form the core microbiota of honey bees (Kwong and Moran, 2016), between the newly emerged versus 48 h old bees was determined by qPCR using bacterial phylotype-specific primers (Table S1). As there were no reported primers that allow differentiation between *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, primers enabling the detection of both species were used.

The qPCR reactions consisted of 5 µl 2× qPCR mix (Brilliant III Ultra-Fast SYBR Green QPCR Mix, Agilent, Santa Clara, CA, USA), 0.25 µl of each forward and reverse primer (20 µmol l⁻¹), 0.5 µl DNA and 4 µl nuclease-free water. qPCR was run on a CFX384 C1000 Touch Real-Time PCR System (Bio-Rad, Hercules, CA, USA). PCR amplification consisted of the following steps: 95°C for 3 min, followed by 40 cycles of 95°C for 20 s, 55°C for 25 s and 72°C for 30 s, and incubation at 72°C for 10 min. After amplification, a dissociation curve was constructed using 81 complete cycles of incubation where the temperature was increased by 0.5°C per cycle, beginning at 55°C and ending at 95°C to verify specificity of the primers.

The fold-difference in the relative DNA concentration following PCR amplification of the *16S rRNA* gene is given as the mean±s.d. Independent *t*-tests were used for analyzing differences in the relative *16S rRNA* sequence levels between the newly emerged versus 48 h old honey bees.

Transcript analysis using reverse transcription

qPCR (qRT-PCR)

qRT-PCR was used for determining the expression of target genes in the RNA samples extracted. The primers used were from previous reports or designed by using primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>) based on the target gene mRNA sequence (Table 1). A qRT-PCR reaction system was set up as follows: 6.25 µl of 2× Brilliant II SYBR Green qRT-PCR 1-Step Master Mix (Agilent), 0.375 µl of forward primer and reverse primer, 0.5 µl of RT/RNase block enzyme mixture, 0.5 µl of extracted RNA and nuclease-free water to a final volume of 12.5 µl. qRT-PCR reactions were run on a CFX384 C1000 Touch Real-

Time PCR System (Bio-Rad). The thermocycler program was as follows: 50°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 59°C for 60 s and 72°C for 60 s, then 72°C extension for 10 min. After amplification, a dissociation curve was constructed using 81 complete cycles of incubation where the temperature was increased by 0.5°C per cycle, beginning at 55°C and ending at 95°C to verify specificity of the primers.

Quantification of DWV positive and negative replicative strands

The total titer and the quantity of the negative strand of DWV were quantified as follows. cDNA synthesis from the extracted RNA was carried out with a random hexamer primer and a tagged DWV-specific primer (5'-AGCCTGCGCACGTGGgaaaccaactctgaggaa-3') using a SuperScript™ III RT-PCR kit (Invitrogen). Then, three pairs of primers – *β-actin* (Table 1) as reference, and a DWV-specific (forward: 5'-CGAAACCAACTTCGAGGAA-3', reverse: 5'-GTGTTGATCCCTGAGGCTTA-3') and DWV negative strand-specific primer pair (tag-sense: 5'-AGCCTGCGCACCGTGG-3' and DWV-antisense: 5'-GTGTGATCCCTGAGGCTTA-3') – were used for qPCR with the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, then 72°C extension for 10 min, and a dissociation melting curve was employed to verify primer specificity.

Data analysis

The comparative Ct ($2^{-\Delta\Delta Ct}$) method was used for comparing the bacterial phylotypes between newly emerged bees and 48 h old workers. The comparative Ct method was also used for calculating the relative expression level of the nutrition-related genes *ilp2*, *tor*, *vg* and *mjrp1–5*, and the immune gene *rel*, and positive and negative strands of DWV (Schmittgen and Livak, 2008). As there were two factors (pollen and antibiotics) in the experimental setup, gene expression data were analyzed via two-way ANOVA using SPSS (PASW Statistics 18, SPSS Inc.).

Survival rates were compared among the treatment groups via SPSS using the Kaplan–Meier method based on the number of dead

Table 1. Primers used for measuring expression levels of *β-actin*, *16S rRNA*, nutrition signal pathway (*tor*, *ilp1*, *vg* and *mjrp1–5*) and immunity (*rel*) genes by qRT-PCR

Gene name	Accession no.	Sequence (5' to 3')	Annealing temperature (°C)	Reference and notes
<i>β-actin</i>	NM_001185146	F: TTGTATGCCAACACTGTCCTTT R: TGGCGGATGATCTTAATTT	59°C	Chen et al., 2005
<i>16S rRNA</i>		F: AGAGTTTGATCCTGGCTCAG R: CTGCTGCCTCCGTAGGAGT	59°C	Powell et al., 2014
<i>vg</i>	NM_001011578.1	F: TCGACAACCTGCGATCAAAGGA R: TGGTCACCGACGATTGGATG	59°C	Schwarz et al., 2016
<i>tor</i>	XM_006566642.3	F: AACAACTGTTGCTGACGGTG R: GTTGCAAGTCCAGGCTTTTTG	59°C	Patel et al., 2007
<i>ilp1</i>	XM_026442143.1	F: CGATAGTCTGGTCCGTTTG R: CAAGCTGAGCATAGCTGCAC	59°C	Wang et al., 2013
<i>rel</i>	XM_026444175.1	F: GCAGTGTGAAGGAGCTGAA R: CCAATTCTGAAAAGCGTCCA	59°C	Schluns and Crozier, 2007
<i>mjrp1</i>	NM_001011579.1	F: AGCAGACGAGAAAGGTGAAGG R: TTGGACTCCTTCGTAATGTATGTCA	59°C	This paper
<i>mjrp2</i>	XM_026443530.1	F: CCTGATTGGTCGTTTGACAGAG R: TGGTCTGCCATGTACACTAAAG	59°C	This paper
<i>mjrp3</i>	NM_001011601.1	F: AACAAAGCGCAGCTGTGAATC R: TGTCTTATCACGCCATCTGTCC	59°C	This paper
<i>mjrp4</i>	NM_001011610.1	F: TAGAGGTGGCGTTGTTCCGAG R: CGAGAAAAGTCTTGTGTGCCA	59°C	This paper
<i>mjrp5</i>	NM_001011599.1	F: CTTGTTGTTGCTGGTCGTG R: GTCATACCACGCCATTGATCG	59°C	This paper

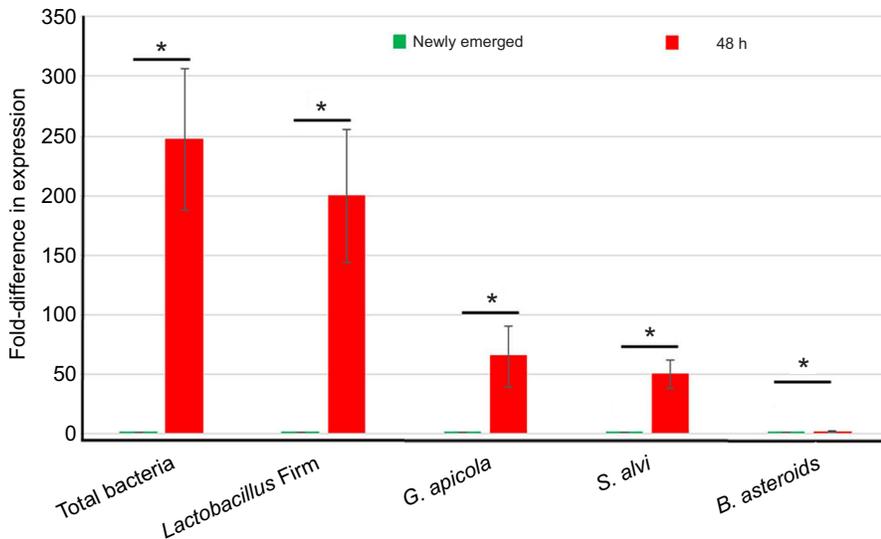


Fig. 1. The relative expression of the 16S rRNA gene in newly emerged bees and 48 h old adult workers. The expression level in total bacteria and its phylotypes (*Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus Firm*-4 and Firm-5 and *Bifidobacterium asteroides*) in newly emerged bees was given a value of 1 and used as a calibrator. The relative abundance of total bacteria and specific bacterial phylotypes in 48 h old honey bees was expressed as an *n*-fold difference relative to the calibrator. * $P < 0.05$.

bees recorded per cage every day. The log-rank was computed to assess the overall homogeneity among the treatments. Pairwise comparisons were carried out using log-rank tests. In all cases, $P < 0.05$ was regarded as statistically significant.

RESULTS

Bacterial phylotypes in the experimental honey bee

The initial state of bacterial phylotypes in experimental honey bees was determined by quantifying the level of DNA encoding bacterial 16S rRNA. Our results showed that the DNA concentration of bacterial 16S rRNA in experimental adult honey bees was 188 times higher than that of newly emerged bees (t -test, $t = -16.349$, $P < 0.001$). Fold-differences in DNA concentration for total bacteria and bacterial species cluster (*Lactobacillus Firm*, *G. apicola*, *S. alvi* and *B. asteroides*) between newly emerged bees and 48 h old honey bees were 247.34, 151.43, 65.24, 50.09 and 2.03, respectively. These values were significantly different (t -test: total bacteria, $t = -4.177$, $P = 0.002$; *Lactobacillus Firm*, $t = 2.516$, $P = 0.026$; *G. apicola*, $t = -2.366$, $P = 0.034$; *S. alvi*, $t = -3.719$, $P = 0.003$; *B. asteroides*, $t = -3.401$, $P = 0.005$) (Fig. 1). The relative percentage of the bacterial phylotypes *Lactobacillus Firm* (4 and 5), *G. apicola*, *S. alvi* and *B. asteroides* was 59.85%, 19.54%, 15.00% and 0.61%, respectively, in 48 h old honey bees, which is in agreement with a previous report by Moran et al. (2012).

Antibiotic disruption of gut bacterial communities

After feeding the bees with antibiotics (groups II and IV), we observed a significant reduction of total gut bacteria as observed by 16S rRNA gene levels (two-way ANOVA, $F = 24.057$, $P < 0.001$) (Fig. 2). The addition of pollen (group II) did not significantly increase the amount of bacteria present ($F = 1.899$, $P = 0.177$), and we observed no interaction between the pollen diet and antibiotic treatment ($F = 1.899$, $P = 0.177$) (Fig. 2).

Antibiotics negatively impact nutritional gene expression and head mass

The expression of *tor* in honey bees from group I and III was significantly higher than that in honey bees from group II and IV (two-way ANOVA, $F = 21.191$, $P < 0.001$) (Fig. 3A). The presence of pollen had no effect on the expression of *tor* (group I versus III) ($F = 0.068$, $P = 0.796$), and there was no interaction between pollen feeding and antibiotic treatment ($F = 0.067$, $P = 0.797$) (Fig. 3A).

Collectively, we observed that it is specifically the antibiotics that influence *tor* expression, not the presence of pollen. A similar pattern was observed for the expression of *ilp1*, with levels in honey bees from group I and III being significantly higher than those in honey bees from group II and IV (two-way ANOVA, $F = 39.408$, $P < 0.001$) (Fig. 3B). However, in this instance, the addition of pollen in the diet significantly increased *ilp1* expression (group I versus III) ($F = 4.634$, $P = 0.038$), and we observed a significant interaction between pollen feeding and antibiotic treatment ($F = 5.133$, $P = 0.030$) (Fig. 3B). The observations suggest that disruption of gut bacteria negatively affects nutrient sensing and signaling. Expression of *vg*, a common marker for overall honey bee health, was significantly higher in pollen-fed honey bees (groups I and II) than in bees that were not supplemented with pollen (two-way ANOVA, $F = 9.693$, $P = 0.004$). Antibiotic treatment (groups II and IV) did not significantly affect the expression level of *vg* ($F = 1.438$, $P = 0.240$), and there was no interaction between pollen feeding and antibiotic treatment ($F = 0.374$, $P = 0.546$) (Fig. 4). In this case pollen could partially compensate for reduced numbers of gut bacteria resulting from antibiotic treatment (group I versus II). Consuming

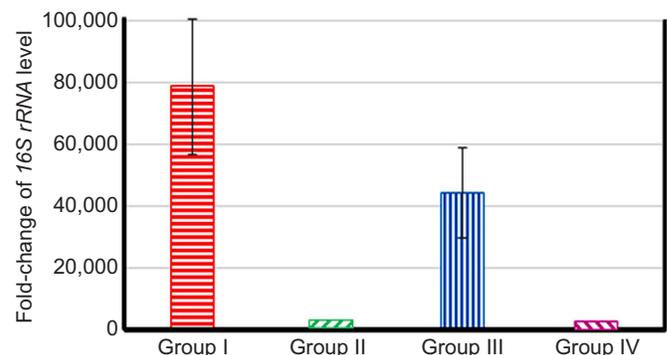


Fig. 2. Effect of antibiotics on the activity of honey bee gut bacteria. Survival of gut bacteria in honey bees from the four groups (group I: pollen control; group II: pollen plus antibiotics; group III: no pollen, no antibiotics; and group IV: antibiotics, no pollen) was determined by the level of 16S rRNA transcripts from gut bacteria using qRT-PCR. The 16S rRNA gene transcript level for gut bacteria of group IV honey bees was the lowest and thereby served as a calibrator. The relative expression of gut bacteria in other groups was calculated by the comparative $2^{-\Delta\Delta Ct}$ method. Data are means \pm s.d. and differences were analyzed by two-way ANOVA.

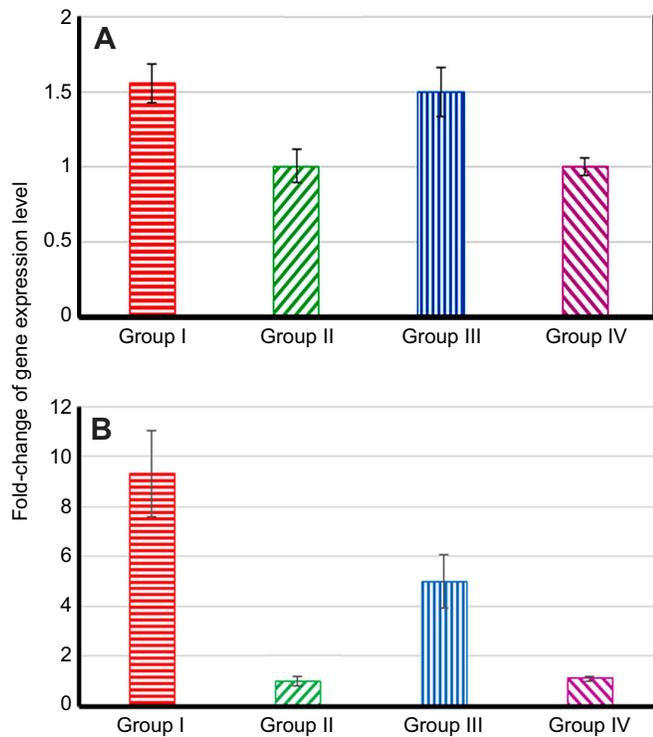


Fig. 3. Effect of pollen diet and antibiotics on the expression of *tor* and *ilp1* in honey bees. (A,B) The relative expression levels of *tor* (A) and *ilp1* (B). Data are mean \pm s.d. expression level relative to that of group IV (*tor*) or group II (*ilp1*) honey bees (lowest expression) and differences were analyzed by two-way ANOVA.

pollen (group I) affected the expression of the *mrjp1*, *mrjp2*, *mrjp4* and *mrjp5* (two-way ANOVA: *mrjp1*, $F=4.788$, $P=0.035$; *mrjp2*, $F=4.842$, $P=0.033$; *mrjp4*, $F=5.913$, $P=0.019$; *mrjp5*, $F=4.285$, $P=0.045$), while antibiotic treatment had a negative effect on *mrjp1*, *mrjp4* and *mrjp5* expression (two-way ANOVA: *mrjp1*, $F=4.158$, $P=0.049$; *mrjp4*, $F=5.484$, $P=0.024$; *mrjp5*, $F=4.252$, $P=0.046$) (Fig. 5). We did observe an interaction between pollen feeding and antibiotic treatment on expression of *mrjp3*, *mrjp4* and *mrjp5*, implying that pollen significantly increased the expression of *mrjp* genes but not in the presence of antibiotics (two-way ANOVA: *mrjp3*, $F=13.303$, $P=0.001$; *mrjp4*, $F=5.229$, $P=0.027$; *mrjp5*, $F=6.256$, $P=0.016$) (Fig. 5). In the case of *mrjp2*, however, the addition of pollen significantly compensated for the effects of antibiotic treatment ($F=4.842$, $P=0.033$) (Fig. 5).

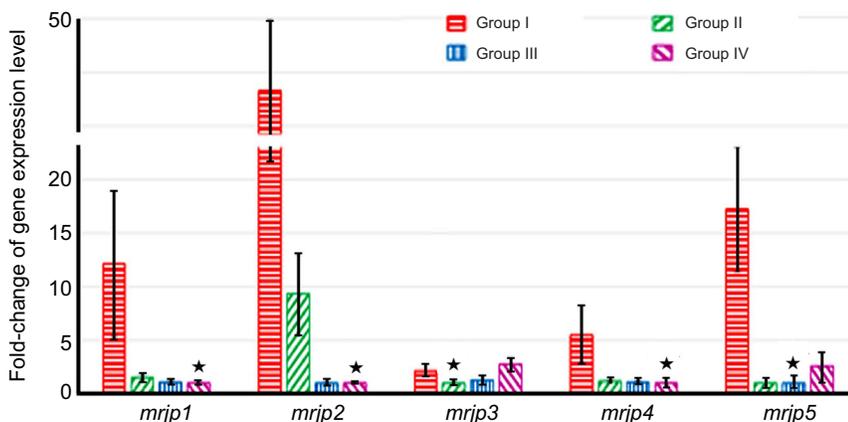


Fig. 5. Effect of pollen diet and antibiotics on the expression of *mrjp1-5* in honey bees. Data are mean \pm s.d. expression level relative to that of honey bees from the indicated group (star, lowest expression) and differences were analyzed by two-way ANOVA.

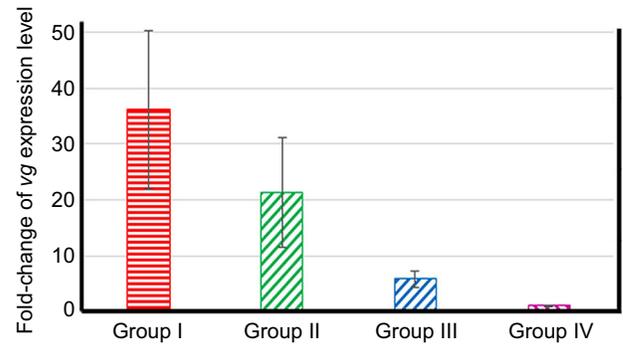


Fig. 4. Effect of pollen diet and antibiotics on the expression of *vg* in honey bees. Data are mean \pm s.d. expression level relative to that of group IV honey bees (lowest expression) and differences in *vg* expression among the honey bees of the four groups were analyzed by two-way ANOVA.

With respect to head mass, honey bees fed pollen (groups I and II) had a significantly greater head mass than those not supplemented with pollen (groups III and IV) (two-way ANOVA, $F=82.412$, $P<0.001$) (Fig. 6). Pollen feeding reduced the effects of antibiotics on HPG development as group II bees had a greater head mass than that of groups III or IV ($F=10.708$, $P=0.002$) (Fig. 6).

Antibiotics suppress immune function and promote DWV replication

Antibiotic treatment (groups II and IV) significantly decreased the expression of immune transcription factor *rel* (two-way ANOVA, $F=10.110$, $P=0.003$). Feeding with pollen did not affect *rel* expression when combined with antibiotic feeding ($F=0.700$, $P=0.409$). There was no interaction between pollen feeding and antibiotic treatment ($F=0.002$, $P=0.961$) (Fig. 7). Conversely, antibiotic treatment (group II and IV) significantly increased both the total level of DWV and negative replicative strand (two-way ANOVA: total level, $F=11.473$, $P=0.002$; negative strand, $F=17.662$, $P<0.001$) (Fig. 8). Consuming pollen did not affect the DWV quantity in terms of both total titer and negative strand (two-way ANOVA: DWV titer, $F=0.314$, $P=0.579$; negative strand, $F=0.011$, $P=0.919$) as DWV titer in group II was similar to that in group IV. There was no observable interaction between pollen feeding and antibiotic treatment (two-way ANOVA: total level, $F=0.658$, $P=0.423$; negative strand, $F=0.567$, $P=0.465$) (Fig. 8).

Antibiotics negatively impact honey bee longevity

Analysis of honey bee lifespan showed that bees fed pollen alone (group I) had the longest survival, while bees receiving antibiotics

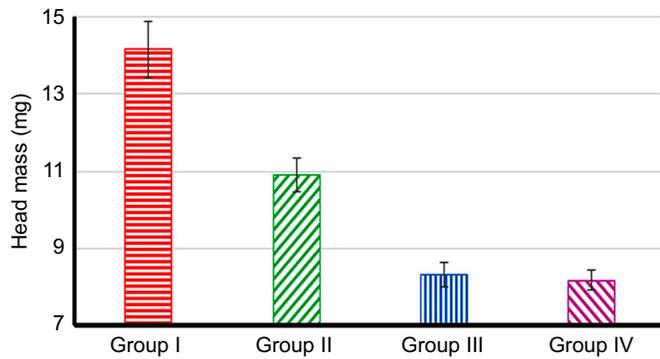


Fig. 6. Effect of pollen diet and antibiotics on honey bee head mass. Data are mean \pm s.d. Differences between the groups were analyzed by two-way ANOVA.

alone (group IV) demonstrated the shortest (log-rank test: group I versus III, $\chi^2=46.607$, $P<0.001$; group II versus IV, $\chi^2=16.871$, $P<0.001$). Comparisons of longevity between treatments with either pollen or sugar syrup in the absence or presence of antibiotics indicated significant decreases in survival when antibiotics were present (log-rank test: group I versus group II, $\chi^2=52.726$, $P<0.001$; group III versus IV, $\chi^2=19.039$, $P<0.001$) (Fig. 9). Additionally, the honey bees from group II had a statistically similar survival rate to those from group III (log-rank test: $\chi^2=0.355$, $P=0.551$) (Fig. 9), implying that pollen may partially counteract the negative effect of antibiotics on honey bee survival.

DISCUSSION

Nutrition affects a variety of aspects related to honey bee biology, physiology and behavior (Ament et al., 2008, 2010; Mutti et al., 2011; Patel et al., 2007; Toth et al., 2005; Wang et al., 2013). Nutritional effects on an organism are driven by the expression of genes involved in metabolism, and by gut bacteria that can impact multiple aspects of health and disease (Butler et al., 2013; Evans and Armstrong, 2006; Glittenberg et al., 2011; Hooper et al., 2012; Kwong et al., 2017; Raymann et al., 2017; Vásquez et al., 2012; Zheng et al., 2017). Additionally, viral infection levels can also be affected by nutritional state and related immune responses (DeGrandi-Hoffman and Chen, 2015). In this study, we sought to comprehensively assay these aspects concomitantly by determining the expression of metabolic and immune genes, viral titer, and the effects on tissue development and lifespan in bees that were nutrient challenged and had gut microbial

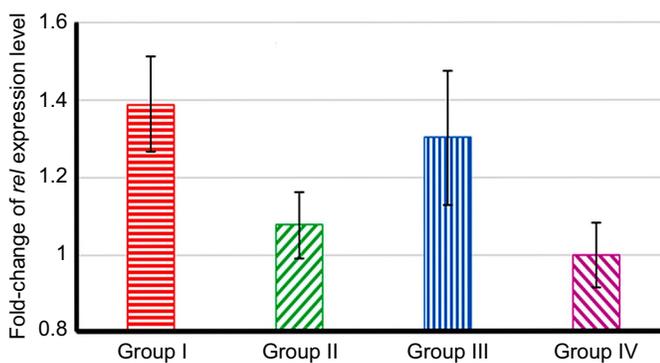


Fig. 7. Effect of pollen diet and antibiotics on the expression of *rel* in honey bees. Data are mean \pm s.d. expression level relative to that of group IV honey bees (lowest expression) and differences in *rel* expression among honey bees of the four groups were analyzed by two-way ANOVA.

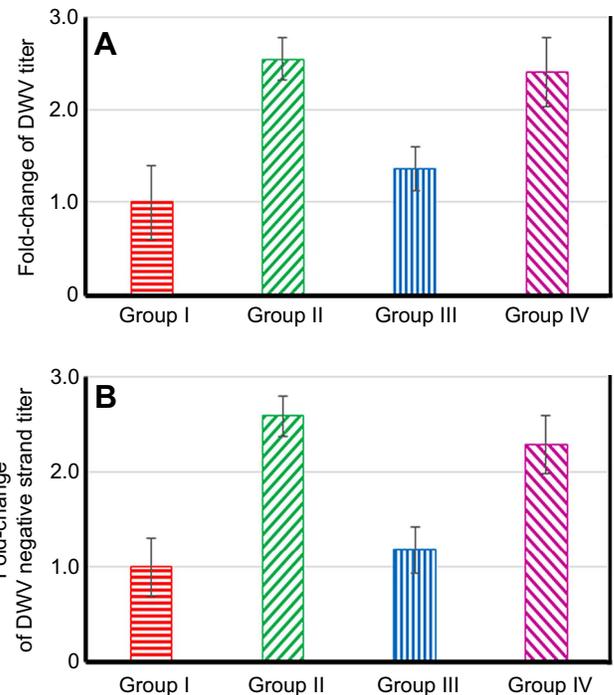


Fig. 8. Effect of pollen diet and antibiotics on deformed wing virus (DWV) titer and its replication activity in honey bees. (A,B) DWV titer (A) and DWV negative strand titer (B) among the different experimental groups. Data are mean \pm s.d. expression level relative to that of group I honey bees (lowest expression) and differences in DWV quantity among honey bees of the four groups were analyzed by two-way ANOVA.

communities compromised by antibiotics. We observed a reduction in gut microbial communities, effects on nutrient and immune gene expression, an increase in the total replicating viral load and a reduction in lifespan and HPG development (estimated by head mass) in bees that were treated with antibiotics and had nutritional deficiencies. These results paint a more thorough picture of the role

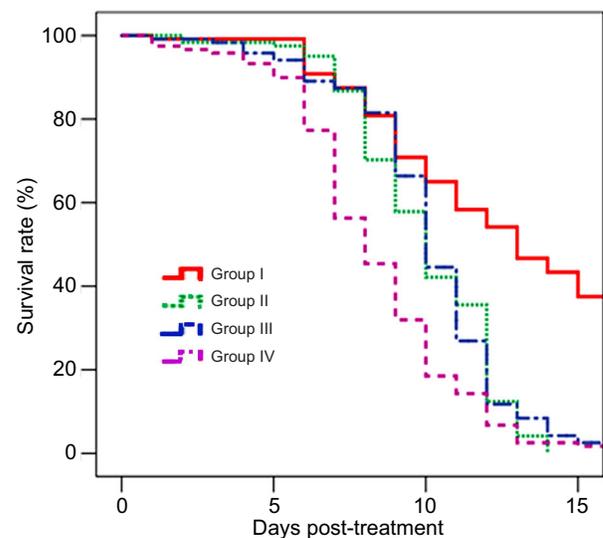


Fig. 9. Effect of pollen diet and antibiotics on the survival rate of honey bees. Survival rate was based on the daily accumulated mortality. Honey bees from group I (control, pollen) had the highest survival rate, while honey bees from group IV (antibiotics, no pollen) had the lowest survival rate. Honey bees from group II (antibiotics plus pollen) had an identical survival rate and time to those from group III (no antibiotics, no pollen).

of the gut microbiome in nutrient processing and immune response, and how it can be affected by antibiotic use to the detriment of the honey bee.

The bacterial species clusters *S. alvi* and *G. apicola*, which are members of Proteobacteria phylum, *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, which are members of the Firmicutes phylum, and *B. asteroides*, which belongs to the Actinobacteria phylum, comprise the major populations of microbiota in adult honey bee gut (Kwong and Moran, 2016). Newly emerged bees acquire them from the hive environment or via contact with nurse bees (Powell et al., 2014). The results from this study and our previous study (Li et al., 2017) clearly show that similar phylotypes of gut microbiota are well established in honey bees after roaming on the surface of a brood frame for 48 h.

Penicillin–streptomycin is an antibiotic combination widely used in *in vitro* cell culture in the lab setting to avoid bacterial infection. Typically, it is not used in beekeeping; ergo, the gut bacteria in honey bees likely presents little resistance to its effects, as was clearly observed in our study (Fig. 2). This result demonstrates that our antibiotic treatment regimen robustly disrupted midgut microbes and provided a background for observing the multitude of its effects on honey bee biology. In practice, however, a number of antibiotics such as lincomycin, tylosin, erythromycin and oxytetracycline are used commonly for the control of American foul brood bacteria, and these drugs have inherent side effects on the development and health of honey bees (Alippi et al., 1999; Elzen et al., 2002; Miyagi et al., 2000; Pettis and Feldlaufer, 2005). It is also important to note that, since 2017, honey bees have been under the Veterinarian Feed Directive, and prescriptions are required for the application of antibiotics in field settings.

Pollen is the primary source of protein for honey bees, and is coupled to honey bee nutritional metabolism. Our results showed that pollen upregulated the expression of *ilp1*, an early nutritional response gene (Fig. 3B). Pollen also increased the expression of *vg*, a hemolymph protein secreted by the fat body, implicated in a variety of biological functions (Fig. 4). *Vg* is regarded as a biomarker of honey bee health, being involved in a number of processes including lifespan (Amdam et al., 2003; Dainat et al., 2012). This result is in agreement with previous research showing that a pollen diet significantly affected the expression of *vg* (Huang et al., 2014). Pollen also increased the expression of *mrjp* genes (Fig. 5), and MRJPs are a key component of royal jelly secreted by the HPG in nursing bees for feeding larvae. Furthermore, honey bees that were not fed pollen (group III and IV) had a lighter head mass than those from the corresponding pollen-fed bees (groups I and II) (Fig. 6). These results clearly show the fundamental role of pollen in honey bee nutritional metabolism and its related physiological characteristics.

Antibiotic treatment and the effect on the gut microbiome decreased the expression of *tor* (Fig. 3A), a downstream nutritional sensor gene determining multiple honey bee biology characteristics such as caste determination. Moreover, antibiotic treatment also decreased the effect of pollen on the expression of other nutrition-related genes, such as *ilp1* and *mrjp* genes, and on head mass. Among the two nutritional genes, *ilp1* is an early ligand for the insulin signaling pathway, mainly responsible for direct sensing of nutrition level (Ihle et al., 2014). *tor* is a downstream kinase of the nutrition pathway, mainly responsible for the later regulation of protein synthesis and autophagy (André and Cota, 2012), and also undergoes regulation by other cellular processes. Pollen ameliorated the negative effects of antibiotics on *ilp1* but not *tor* (Fig. 3). It is possible that the phosphorylation state of Tor may play a role in this particular circumstance.

Previous studies showed that gut bacteria can aid in honey bee nutrition (Engel et al., 2012; Kwong and Moran, 2015; Saraiva et al., 2015). Nutritional regulation by gut bacteria has also been reported in other animals like fruit flies, aphids and humans (Akman Gündüz and Douglas, 2009; Bäckhed et al., 2005; Douglas, 2015; Engel and Moran, 2013; Nicholson et al., 2012; Wong et al., 2014; Zientz et al., 2004), suggesting a universal gut requirement for bacteria within eukaryotic hosts. Gut bacteria in humans can affect nutrition metabolism through several cellular factors released by gut bacteria that influence host metabolism (Ramakrishna, 2013). Recent work in honey bees has elucidated some of the effects of antibiotic perturbation on gut microbial communities, including reduced survivorship (Raymann et al., 2017).

Immunity is another important factor affecting honey bee overall health and survival (Amdam et al., 2004; Dainat et al., 2012). Honey bees have many similar components to *Drosophila* to counter various infections through AMPs (Aggarwal and Silverman, 2008; Evans et al., 2006; Lemaitre and Hoffmann, 2007). The transcription factor *rel* is directly upstream of AMP gene expression in the immunodeficiency (IMD) pathway (Schlüns and Crozier, 2007). Our study showed that antibiotic treatment decreased the expression of *rel*, which leads to the down-regulation of AMPs, as we previously reported (Li et al., 2017). Modulation of host immunity by gut bacteria to the benefit of the host has also been well reported (reviewed in Deitch et al., 1991; Ha et al., 2005; Hooper et al., 2012; Kamada et al., 2013; MacDonald and Monteleone, 2005).

DWV exists in all stages of the honey bee, typically as a latent non-pathogenic infection. However, under some stressful conditions, DWV can replicate quickly, which shortens honey bee lifespan and ultimately leads to whole colony losses (Deitch et al., 1991; Genersch and Aubert, 2010; Tantillo et al., 2015). Our results showed that gut bacteria disruption by antibiotics results in a change from a latent DWV infection to an actively replicating one, suggesting that gut bacteria play an extremely important role in preventing DWV replication in honey bees. This phenomenon has also been well documented for the interaction between the Dengue RNA virus and its vector *Aedes aegypti* mosquito (Ramirez et al., 2012; Xi et al., 2008). Additionally, Influenza A virus is more successful in humans with antibiotic-compromised digestive systems (Tripathi et al., 2015).

Life span is a comprehensive reflection of honey bee health. Nutrition and immunity are two of the most critical factors affecting honey bee lifespan. Besides the decrease in nutritional and immune levels, antibiotic treatment also significantly shortened the lifespan of honey bees (group II and IV) compared with that of the non-treated honey bees (group I and III). Pollen, however, increased the lifespan of honey bees (group I and II). Similar conclusions have previously been drawn (Raymann et al., 2017). Honey bees from group II (antibiotics plus pollen) shared an identical lifespan with honey bees from group III (no antibiotics, no pollen) (Fig. 9). Antibiotic treatment decreased honey bee nutrition metabolism and immunity in our study. Even when no pollen was supplied (group III), nutrition metabolism and immunity were not compromised, perhaps because gut bacterial communities were present. In fact, the lifespans of bees with and without pollen were similar if they were not fed antibiotics and the gut microbiome was undisturbed. Taken together, these results suggest that both pollen and gut microbes are needed to promote honey bee health.

Conclusion

Honey bees harbor numerous obligate symbionts in the gut. These bacteria provide multiple biological functions that support

metabolism and immunity and affect tissue development and lifespan. Herein, we demonstrated that antibiotic treatment that compromises gut microbial communities impairs metabolism, weakens immunity, increases DWV titer and shortens the lifespan of the honey bee. Consuming pollen could counteract, in part, the negative effects caused by antibiotics, but the total health benefits of a pollen diet could not be realized if there was a reduction in gut microbial communities.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.L., M.C.H., Y.C.; Methodology: J.L., M.C.H., Y.C.; Validation: J.L., M.C.H., Y.C.; Formal analysis: J.L., M.C.H., Y.C.; Investigation: J.L., M.C.H., J.D.E., W.L., C.R.-G., G.D.-H., Y.Z., S.H., Z.L., M. Hamilton, Y.C.; Resources: R.R., G.D.-H., Y.C.; Data curation: J.L., M.C.H., Y.C.; Writing - original draft: J.L., M.C.H., Y.C.; Writing - review & editing: J.L., M.C.H., R.R., G.D.-H., Y.C.; Supervision: J.D.E., Y.C.; Funding acquisition: J.L., Y.C.

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Supplementary information

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