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Host-Microbial Interactions | Full-Length Text

Queen bee gut microbiota extends honeybee lifespan by inhibiting insulin signaling

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ABSTRACT Queen and worker bees are natural models for aging research, as their lifespans vary considerably independent of genetic variation. Investigating the reasons why queens live longer than workers is of great significance for research on the universal processes of aging in animals. The gut microbiome has received attention as a vital regulator of host health, while its precise role in honeybee aging needs further investigation. The effects and mechanisms behind the relationship between gut microbiota and worker lifespan were measured by transplanting queen bee gut bacteria (QG) and worker bee gut bacteria (WG) into microbiota-free (MF) workers. The transplantation of QG to MF bees significantly extended the workers' lifespans compared with MF and WG bees. Untargeted metabolomics identified 49 lifespan-related differential metabolites, and Kyoto Encyclopedia of Genes and Genomes analysis of these revealed three lifespan-related metabolic pathways: insulin/insulin-like growth factor signaling, immune, and ketone body metabolism pathways. Further verification showed that QG inhibited the expression of insulin-like peptides (ILPs), and the expression of ILPs was lower in natural queens than in natural workers. QG transplantation also stimulated the expression of antioxidant genes and lowered oxidative damage products in natural queen bees. However, gut microbiota transplantation failed to mimic the immune properties and ketone body metabolism profiles of natural queens and workers. Concisely, QG could increase the antioxidant capacity to extend lifespan by inhibiting insulin signaling. These findings may help determine the mechanisms behind queen longevity and provide further insights into the role of gut symbionts.

IMPORTANCE Queen and worker bees share the same genetic background but have vastly different lifespans. The gut microbiome regulates host health, suggesting that differences in lifespan between queen and worker bees could be related to gut bacteria. Herein, we used an innovative method to transplant gut microbiota from adult queen or worker bees to microbiota-free bees. The transplantation of queen gut microbiota to microbiota-free bees extended their lifespan. Insulin/insulin-like growth factor signaling, a highly conserved metabolic pathway related to lifespan, displayed identical expression profiles in natural queen bees and microbiota-free bees transplanted with queen microbiota. This finding significantly expands our understanding of the relationships between intestinal bacteria, host health, and the biology of aging.

KEYWORDS queen bees, worker bees, microbiota, lifespan, insulin/insulin-like growth factor signaling, antioxidants

Female honeybee embryos can develop into either worker or queen bees depending on what they are fed, thus revealing that queen and worker bees share a common genome. However, the maximum lifespan of queen bees is 20 times longer than that of worker bees. Queen bees generally live 2–5 years, though replaced frequently during commercial production (1). The longest lifespan of a queen ever recorded was 8 years (2).

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However, worker bees only live up to ~140 days during winter, when their activity levels are low (3). The queen and worker bees, despite their lack of genetic variation, provide distinct natural aging models with significant differences in lifespan.

Uncovering the mechanisms behind this intriguing phenomenon attracts interest both at a fundamental level and for broader research on aging. A widely recognized factor is the different nutritional regimes of queen and worker bees. Queen bees are fed royal jelly (RJ) throughout their lives, whereas worker bees are fed RJ for only a short period of time during the larval stage (4–6). Previous research has demonstrated that feeding workers RJ extended their lifespan (7). This raises the question on the molecular mechanisms by which nutrition or other factors extend the lifespan of queen bees. Advances in this field have suggested that there may be three main molecular mechanisms behind this.

First, the insulin/insulin-like growth factor signaling (IIS) pathway is highly conserved in animals and model organisms, such as yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice (8, 9). Inhibition of IIS has been shown to extend lifespan, with a similar pattern identified in honeybees. IIS in long-lived queen bees has been shown to be inhibited relative to that in shorter-lived worker bees (10).

Second, oxidative damage and antioxidant capabilities are thought to be involved. The oxidative stress theory, currently the most accepted mechanistic theory of aging, suggests that reactive oxygen species (ROS) produced by healthy cellular metabolism create oxidative damage through attacking macromolecules (e.g., nucleic acids, proteins, and lipids). This progressive accumulation of oxidative damage over time has been shown to cause aging and ultimately determine the lifespan (11). A comparative study on antioxidant systems between queen and worker bees during the larval stage demonstrated that queen bees had higher expression of antioxidant genes (*MnSOD*, *CuZnSOD*, *catalase*, and *Gst1*) than worker bees, whereas no significant differences were found for protein carbonylation levels between castes (12). Notably, antioxidant abilities have not been adequately explored and compared between workers and queens (the female castes). Intriguingly, IIS and antioxidant pathways were found to be cross-linked in their regulation of longevity. Decreased IIS could diminish the phosphorylation of forkhead box O transcription factor (FOXO), leading FOXO proteins to remain in the nucleus. Nuclear FOXO protein facilitates the transcription of antioxidant genes, including *superoxide dismutase 2* (*SOD2*), *catalase*, *glutathione peroxidase* (*GSH-PX*), and others (13).

Third, immunosenescence, referring to the deterioration of immunocompetence with age, is a feature common in aging processes in animals (14). A similar immunosenescence pattern has been found in social insects (15, 16). Melanization is a form of humoral immunity in insects. Phenoloxidase (PO) is a core enzyme that catalyzes melanization, and the highest expression levels of PO in queen bees were shown to be two times as high as those in worker bees (17). In another social insect, the black garden ant, RNA sequencing results suggested that the expression of immune-related genes was highest in the queen ant at 1 day of age (18). These studies suggested that longer-lived queen social insects may have higher immunocompetence than shorter-lived social insect workers.

There may be further factors related to the extended lifespan of queen bees compared with worker bees. Studies have demonstrated that gut commensal microbiota provide a unique approach to gain insights into bee health and disease. It has been proposed that the gut microbiota represent an "extended immune phenotype" in addition to the host's immune system (19), suggesting that bee health is significantly correlated with their gut microbiota. A study by Anderson et al. (20) suggested that queen bee gut microbiota were refined with age. Our previous study identified that changes in gut microbial composition, such as increasing gut Acetobacteraceae abundance, via dietary modulation could extend the longevity of overwintering bees (21). Moreover, compared with worker bees, one of the dominant queen gut bacteria types was the Acetobacteraceae family (22). The above study suggested that gut

microbiota are at least one of the factors causing queens to live longer than workers. Furthermore, studies in nematodes (23), fruit flies (24), mice (25, 26), and humans (27, 28) have demonstrated that gut microbiota regulate aging and longevity. For example, the lifespan of progeroid mice was extended by transplanting fecal microbiota from healthy young mice (25). Thus, we suspected that different gut symbionts between queen and worker bees would contribute to the differences in adult longevity between the two castes. In this study, this hypothesis was tested by transplanting queen bee gut bacteria (QG) and worker bee gut bacteria (WG) into microbiota-free (MF) bees.

MATERIALS AND METHODS

Establishment of microbiota-free honeybee model

The process for obtaining MF honeybees was performed according to the protocol described by Powell et al. (29). Queen bees were confined on empty combs to lay eggs for 12 h with the use of a queen spawning controller (Changge Jihong Beekeeping Equipment Co., Henan, China). Subsequently, the comb was transferred to the super-structure, and the queen bees were kept in the lower hive chamber. Late-stage pupae (24–36 h before eclosion) were transferred to sterile culture plates in the laboratory for eclosion in a sterile incubator (35°C ± 1°C, 57% ± 10% relative humidity, in darkness). The gastrointestinal tract of honeybee pupae is considered almost sterile, as gastrointestinal emptying occurs during the transition from larva to pupa. Thus, the MF model was induced by transferring MF pupae to a sterile environment for eclosion. Total numbers of gut bacteria were quantified using culture-based enumeration. Each honeybee was surface sterilized in 75% ethanol for 1–2 min and washed in sterile phosphate-buffered saline (PBS). The intestines were dissected and homogenized in 150 µL of sterile PBS. Intestinal homogenates were plated onto Luria-Bertani (LB) agar plates and grown at 30°C for 48 h. Subsequently, the colony-forming units on the plate were counted (Fig. S1).

Gut bacteria transplantation

Synchronous female eggs were obtained by confining queen bees to an empty comb to lay eggs for 12 h in the natural hive environment. When the eggs hatched into 1-day-old larvae, some larvae were transferred to artificial queen bee cells for breeding queen bees, while others were left in the original worker cells for breeding worker bees. The sealed brood combs were placed into a gauze-covered isolator, which can isolate newly emerged worker bees from other bees, and placed in the colony until emergence. The gauze-covered isolator was a cage with many grids, which was just big enough to hold a standard nest spleen. The grids can be closed off when it is necessary to prevent workers from entering or leaving the cage. After emergence, the newly emerged worker bees in the gauze-covered isolator were marked on the thorax using a red marker pen (Mitsubishi Pencil, Tokyo, Japan). The red-marked newly emerged bees were released from the isolator into the original colony and allowed free contact with other bees to establish the normal gut symbiotic bacteria. The sealed queen cells were transported to colonies that did not have a queen. The emerged queens were routinely fed by worker bees in a natural hive to establish normal gut symbiotic bacteria. Queens and 7-day-old worker bees were sampled and dissected as described for the MF bees to obtain the entire gastrointestinal tract. Gastrointestinal tissue from queen or worker bees was then placed into a sterile physiological 0.9% saline solution and mechanically broken with sterile ophthalmic scissors into 1 mm^3 chunks. The tissue suspension was filtered with a No. 40 mesh sieve (aperture diameter: 0.38 mm) to remove the intestinal tissue fragments, and the intestinal tissue fragments were rinsed repeatedly to obtain more intestinal contents. Finally, a 10% gut content suspension was prepared as the mother liquid with a physiological salt solution.

Following this, 450 1-day-old MF bees were randomly assigned to three sterile wooden cages (10 × 7 × 8 cm; 150 bees per cage). One group of MF bees was fed

a sterile 50% sucrose solution supplemented with 10% WG-suspension mother liquid (hereafter referred to as WG bees; final concentration of worker bee gut homogenate was 1%); Other group of MF bees was fed a sterile 50% sucrose solution supplemented with 10% QG mother liquid (hereafter QG bees; the final concentration of queen bee gut homogenate was 1%); CK bees, as a control, were fed a sterile 50% sucrose solution supplemented with 10% vehicle solution. All bees were allowed *ad libitum* access to sterile food and water. All wooden cages and utensils were autoclaved to ensure sterility. Bee cages were kept in a sterile incubator (32°C ± 1°C, 57% ± 10% relative humidity, in darkness) in a sterile room. All experiments were repeated in triplicate.

Assays for percent survival, average daily food intake, body weight, and body nutrient composition

The number of dead bees in each cage was recorded daily, and the dead bees were removed. The data were analyzed using Kaplan–Meier survival curves. The intake of sucrose solution (volume) in each cage was recorded daily for 15 days. The total intake of sucrose solution was multiplied by 0.5 (concentration of sucrose solution) and then divided by the number of bees in each cage to obtain the daily food intake of each bee, and the 15-day average daily food intake (ADFI) was calculated. Whole-body wet weights were measured using 10–30 bees from each cage using an electric balance sensitive to 0.0001 g at 1, 9, and 15 days of age. After weighing, 9-day-old bees were used to measure nutrient composition. Bees were dried at 105°C and weighed (dry weight). Dry matter content was obtained by dividing the dry weight by the wet weight. The dried samples were ground to powder for the determination of crude protein and fat. Then, 0.1 g of dry sample powder was placed into a tube containing 0.4 g $CuSO_4{\cdot}5H_2O$ (99%), 6 g K_2SO_4 (99%), and 10 mL H_2SO_4 (98%) (Tianjin Kaitong Chemical Reagent Co., Ltd., Tianjin, China). The samples were digested for 5 h. After digestion, the crude protein content was determined using an automatic Kjeldahl nitrogen tester (Kjeltec 8400; FOSS A/S, Hillerød, Denmark). A further 0.1 g of dry sample powder was used to determine the crude fat content using a Soxhlet extractor. The sample powder was re-dried and re-weighed after extraction, and the crude fat content was obtained as the percentage of lipid loss in the original dry sample powder (30).

RNA extraction and quantitative real-time reverse transcription PCR

Ten 9-day-old worker bees in the respective group were randomly employed for quantitative real-time reverse transcription PCR (qRT-PCR). Total RNA was extracted from individual samples using an RNAiso Plus Kit (Takara, Beijing, China) and treated with RNase-free DNase I to remove the genomic DNA. Subsequently, cDNA was immediately synthesized from 1 ng of the respective RNA sample using a reverse transcription Kit (Takara, Beijing, China). The mRNA expression levels of target genes were measured using qRT-PCR (7500 Real-time System, ABI, USA) in a reaction volume of 20 µL with a SYBR PrimeScript RT-PCR Kit (Takara, Beijing, China). The following cycling conditions were used: 5 min at 95°C (pre-incubation), 10 s at 95°C (denaturation), 20 s at 60°C (annealing), and 30 s at 72°C (extension). Ten bees per cage were used for the qRT-PCR analysis. Three technical replicates were performed for each sample. The β-actin gene (XM_017065464) served as the reference gene. Invitrogen was contracted to synthesize the primers for the target genes, as listed in Table 1. Relative gene expression levels were obtained using the $2^{-\Delta\Delta Ct}$ method.

Assays for oxidative damage products

Samples were weighed and homogenized in PBS (volume:weight ratio, 9:1) and protease inhibitor was added. The suspension was sonicated with an ultrasonic cell disrupter, and the homogenates were then centrifuged for 5 min at 5,000 × *g*. Half of the supernatant was tested for total protein using a BCA Protein Assay Kit (Beyotime, Shanghai, China), and the other half of the supernatant was used for detecting ROS (Insect ROS ELISA Kit,

TABLE 1 Sequences of primers for RT-PCR

Genes	Primer sequences	References
ILP1	F′-GCTCAGGCTGTGCTCGAAAAGT	
	R′-CGTTGTATCCACGACCCTTGC	(10)
InR1	F′-ACGGGATGGCCTACTTGGAG	
	R′-GGAAACCATGCAATTCCTCG	(10)
Catalase	F′-GGCGGCTGAATTAAGTGCTA	
	R′-TTGCGTTGTGTTGGAGTCAT	(31)
GST	F′-CAATTTGATGAACGGGGAAC	
	R′-GCCGTACCGATGTTTTCGTA	(31)
Cyt B	F′-CCAACTCATATTAAACCTGAATG	
	R′-CCGATTACACCTCCTAATTTATT	(32)
Cyt C	F′-CACAAAGTAGGACCTAATCTTTATGGAGTA	
	R′-TCCTTTATTCGCATCTGTGTAGCT	(32)
SOD1	F′-GTCGTTCCGTGTAGTCGAGAA	
	R′-TCCTTTGACTTCACCCTGAAGA	(32)
SOD2	F′-GGTGGTGGTCATTTGAATCATTC	
	R′-AAGAAGTGCAGCGTCTGGTTTAC	(32)
Imd	F′-TGTTAACGACCGATGCAAAA	
	R′-CATCGCTCTTTTCGGATGTT	(33)
Relish	F′-GATGCAGAAGATGAAAAAGCAG	
	R′-TGAACACATTTCGTTTGTTGTTT	(33)
Phenoloxidase	F′-AGATGGCATGCATTTGTTGA	
	R′-CCACGCTCGTCTTCTTTAGG	(33)
Hymenoptaecin	F′-CTCTTCTGTGCCGTTGCATA	
	R′-GCGTCTCCTGTCATTCCATT	(33)
Defensin	F′-TGCGCTGCTAACTGTCTCAG	
	R′-AATGGCACTTAACCGAAACG	(33)
Abaecin	F′-CAGCATTCGCATACGTACCA	
	R′-GACCAGGAAACGTTGGAAAC	(33)
Apidaecin	F′-TTTTGCCTTAGCAATTCTTGTTG	
	R′-GTAGGTCGAGTAGGCGGATCT	(34)
HMG-S	F′-ACAACTAATGCTTGCTATGGAGGT	
	R′-ATCTGCCATCCCAAGCACTG	From this study
HMG-L	F′-GCTAGCCTTGAATGTGGTATAAGAACT	
	R′-TTTCCAGAAGCTCCAAGTGCA	From this study
β-actin	F′-CCGTGATTTGACTGACTACCT	
	R′-AGTTGCCATTTCCTGTTC	(35)

Sino Best, Shanghai, China), malondialdehyde (MDA; Insect MDA ELISA Kit, Sino Best), 4-hydroxynonenal acid (4-HNE; Insect 4-HNE ELISA Kit, Sino Best), protein carbonyls (PC; Insect PC ELISA Kit, Sino Best), and 8-hydroxydeoxyguanosine (8-OHDG; Insect 8-OHdG ELISA Kit, Sino Best).

Microbiome analysis

Twenty 9-day-old honeybees per cage were collected for intestine collection. Honeybees were anesthetized on ice and washed with 75% ethanol before dissection. The head was fixed, and the entire intestine was removed by pulling the stinger with the use of sterile dissecting forceps. The entire procedure was performed under aseptic conditions. Six samples were taken from each treatment group, and each sample was a mixture of the guts of 10 honeybees. Microbial DNA was extracted using a HiPure Stool DNA Kit (Magen Biotech, Guangzhou, China) in accordance with the manufacturer's instructions. The DNA concentration, purity, and integrity were verified using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) ($1.8 < A_{260}/A_{280} < 2.0$) and 1% agarose gels. As

revealed by the quality control results, four samples that did not satisfy the sequencing requirements were removed. Fourteen samples (CK, $N = 4$; WG, $N = 5$; and QG, $N = 5$) were employed for 16S rDNA sequencing (Genedenovo company, Guangzhou, China). The 16S rDNA V3–V4 regions of the ribosomal RNA genes were amplified using PCR with the TransGen High-Fidelity PCR SuperMix (TransGen Biotech, Beijing, China) using the primers: 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTATCTAAT). The purified amplicons were paired-end sequenced (Novaseq 6000, Illumina, San Diego, CA, USA) to generate 250-bp paired-end reads using Novaseq 6000 SP Reagent Kit (Illumina, San Diego, CA, USA). A bioinformatics analysis of 16S rDNA sequencing data was performed in accordance with the method of Wang et al. (35). Raw reads were further filtered using FASTP (v0.18.0), and paired-end clean reads were merged as raw tags using FLASH (v1.2.11) with a minimum overlap of 10 bp and a mismatch error rate of 2%. Noisy sequences of raw tags were filtered to obtain high-quality clean tags, which were clustered into operational taxonomic units of ≥97% similarity using the UPARSE (v9.2.64) pipeline. The representative operational taxonomic unit sequences were classified into taxonomic categories based on the SILVA database (v132).

Untargeted metabolomics

Ten intact guts (~150 mg) were quickly dissected and stored at −80°C. Eight biological replicates from each treatment group were shipped on dry ice to Gene Denovo Biotechnology (Guangzhou, China) for metabolomics analysis. The samples were added to 1 mL of extraction solvent (acetonitrile:isopropanol:H_2O, 2:2:1, vol/vol/vol) and homogenized for 30 s for metabolite extraction. Subsequently, the mixture was centrifuged for 15 min (14,000 *g* at 4°C). Next, the supernatant was dried in a vacuum centrifuge, and the samples were resuspended in 100 µL of acetonitrile/water (1:1, vol/vol) solvent for liquid chromagraphy-mass spectrometry (LC-MS) analysis. The samples were separated by hydrophilic interaction liquid chromatography using a 2.1 × 100-mm ACQUITY UPLC BEH 1.7-µm column (Waters Corporation, Milford, MA, USA). The column temperature was 25 °C, the flow rate was 0.5 mL/min, and the injection volume was 2 µL. The mobile phase comprised A (25 mM ammonium acetate and 25 mM ammonium hydroxide in water) and B (acetonitrile). A 2.1-mm × 100-mm ACQUITY UPLC HSS T3 1.8-µm column (Waters Corporation) was used for reversed-phase liquid chromatography separation. A 2-µL aliquot of the respective sample was injected. The first- and second-order spectrograms of samples were collected using an AB Triple TOF 6600 mass spectrometer (MS). For MS-only acquisition, the instrument was set to acquire over the *m/z* range of 60–1000 Da, and the accumulation time for the time-of-flight MS scan was set to 0.20 s/spectrum. For auto MS/MS acquisition, the instrument was set to acquire over the *m/z* range of 25–1,000 Da, and the accumulation time for the product ion scan was set to 0.05 s/spectra. The product ion scan was acquired using information-dependent acquisition with a high-sensitivity mode.

Untargeted metabolomics data analysis

Raw data were converted into the mzXML format using Proteowizard software (v3.0.8789). The data were further processed through baseline filtration, peak identification, peak filtration, and peak alignment using the XCMS R package (v3.1.3) to obtain the data matrix (e.g., mass-to-charge ratio, retention time, and peak intensity). Metabolites were identified based on exact molecular weight and were then matched against common metabolite databases including METLIN (http://metlin.scripps.edu), Massbank (http://www.massbank.jp/), and mzCloud (https://www.mzcloud.org). Orthogonal projections to latent structures-discriminant analysis, which is an extension of partial least squares-discriminant analysis, was applied for the comparison of groups using the R package ropls (36) (http://www.r-project.org/). Variable importance in projection (VIP) scores of the orthogonal projections to latent structures model were applied to rank the metabolites that best distinguished between the two groups. The VIP threshold was set to 1. In addition, the Student's *t*-test was used as a univariate analysis for screening

differential metabolites (DMs). Those with a *t*-test *P*-value < 0.05 and VIP ≥ 1 were considered DMs between two groups. The abundance of DMs in the same group was normalized using *z*-scores. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a major public database that includes not only genes but also metabolites. Metabolites were mapped to KEGG metabolic pathways for annotation and enrichment analysis. Pathway enrichment analysis identified significantly enriched metabolic pathways or signal transduction pathways in differential metabolites compared with the whole background. The rich factor (RF) was calculated as the number of background metabolites divided by the number of DMs. The calculated *P*-values were false discovery rate corrected, with a threshold of 0.05. Pathways meeting these conditions (FDR ≤ 0.05 or RF > 0.5) were defined as significantly enriched pathways.

Evaluating the effects of targeted metabolites on bee lifespan

Five hundred 1-day-old MF bees from the same colony were randomly assigned to five groups (100 bees/group). The control group was fed a sterile 50% sucrose solution. The acetoacetic acid (AA) group was fed 8, 4, 2, or 1 mg/mL AA in 50% sucrose solution. The number of dead bees in each cage was recorded daily, and the dead bees were removed from the cages. The data were analyzed using Kaplan–Meier survival curves.

Total hemocyte concentration

Bees were anesthetized with CO_2, and their hemolymph was collected. About 10 µL of hemolymph was siphoned from the thorax using a microcapillary tube and was diluted threefold in PBS (hemolymph:PBS ratio = 1:2). Before collecting hemolymph, the abdomen was removed using tweezers to remove the intestines, thereby avoiding cross-contamination from the gut. Trypan blue dye (0.4%) was added to the diluted hemolymph at a ratio of 1:9, followed by homogenization. The number of hemocytes was counted directly in a Neubauer hemocytometry chamber under a microscope.

Statistical analyses

SAS (v9.1; SAS Institute Inc., Cary, NC, USA) was used for data analysis. For the comparison of values across three groups, data distributions were analyzed for normality. Levene's test for homogeneity of variance was performed. Differences in normally distributed values among the three groups were analyzed using the one-way analysis of variance followed by Duncan's multiple range test. Principal coordinates analysis for the gut microbiomes was performed using the Vegan package in R (37) (v2.5.3). Data are expressed as mean ± standard error of the mean. The log-rank (Mantel–Cox) test and log-rank test for trend were used to compare survival curves. Differences were considered statistically significant with *P*-values < 0.05.

RESULTS

Lifespan was extended by queen bee gut microbiota transplantation to microbiota-free bees

The lifespans of QG, WG, and CK (control) bees are illustrated by Kaplan–Meier survival curves in Fig. 1A. Compared with the CK and WG groups, the survival curve of the QG group was significantly shifted to the right ($P < 0.05$). The transplantation of WG to MF bees, compared with CK bees, shifted the Kaplan–Meier survival curve to the right slightly but non-significantly ($P > 0.05$). The strong link between food restriction and longevity was well documented. Thus, the effects of gut microbiota transplantation on body weight, ADFI, and body nutrient composition were assessed (Fig. 1). QG or WG transplantation into MF bees did not affect the adult bees' body weight ($P > 0.05$). Compared with the CK group, QG and WG transplantation significantly increased ADFI ($P < 0.01$) and QG transplantation increased the dry weight ($P < 0.05$). Interestingly, gut microbiota transplantation, with QG or WG, changed the correlation between ADFI and lifespan. In the CK group (MF bees), a significant positive correlation was found between

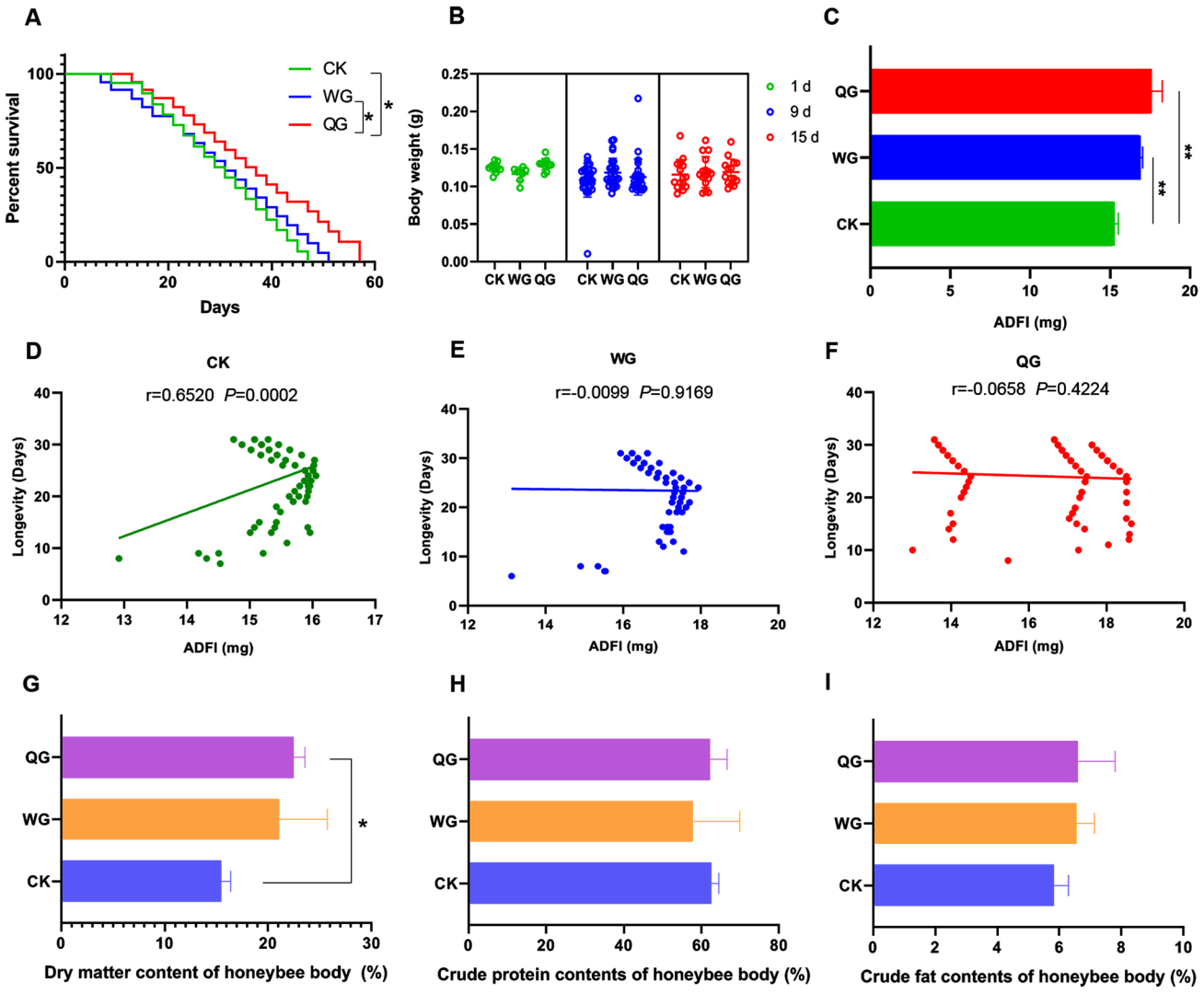

FIG 1 The effects of WG and QG transplantation on lifespan, body weight, ADFI, and body nutrient composition. (A) Lifespan. (B) Body weight. (C) ADFI. (D–F) Correlation between lifespan and ADFI in CK, WG, and QG groups. (G–I) Nutrient composition.

ADFI and lifespan (r = 0.6520 and P = 0.0002). However, microbiota transplantation (QG or WG) turned the relationship between feed intake and life expectancy into a negative correlation trend (P > 0.05). These results preliminarily suggested that gut bacteria may affect longevity by regulating food intake.

Gut microbiota transplantation reshaped the gut microbiota of MF bees

Principal coordinate analysis based on Bray–Curtis similarity suggested that QG or WG transplantation reshaped the gut microbiota structure of MF bees (Fig. 2A). The gut of MF bees in the CK group was expected to be free of microbes, but small amounts of microbiota were still detected. There may be two reasons for this. First, MF bees produced using the method of transferring late-stage pupae are not completely sterile but are relatively sterile compared with newly emerged worker bees with normal social contact. Second, a small amount of microbiota from WG and QG may have been transferred through airborne transmission to the CK group, as the CK group was reared in the same incubator as the WG and QG groups. The gut microbiome of MF bees in the CK group did not have much variety, with more than 98% of their intestinal bacteria being from the Proteobacteria phylum. Although the dominant bacteria in the WG and QG

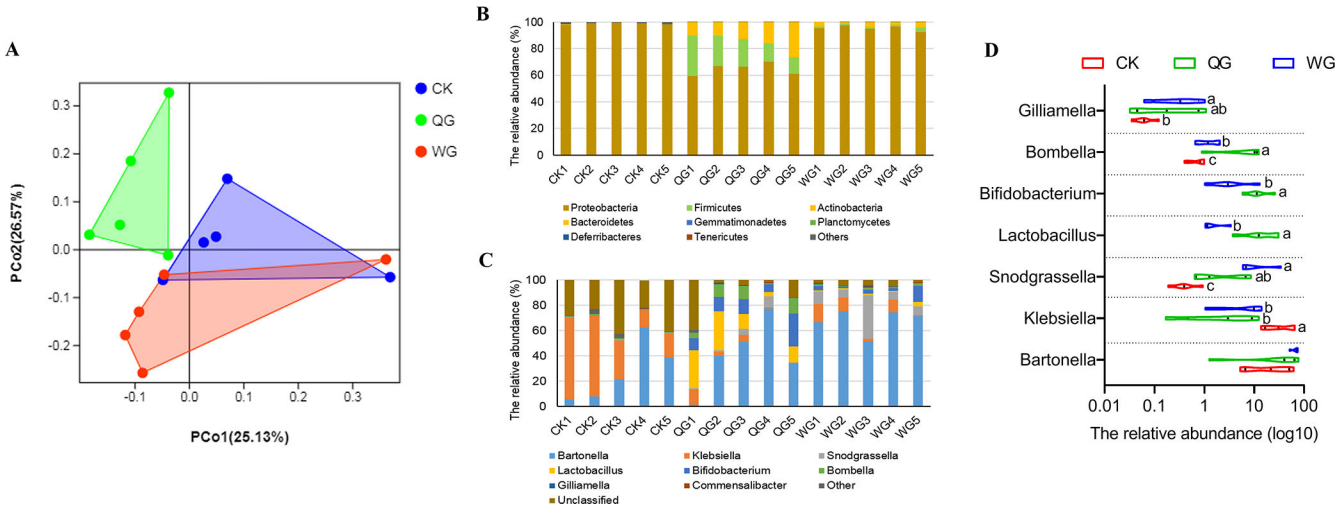

FIG 2 Gut microbiota composition. (A) Principal coordinate analysis plot based on the Bray–Curtis similarity for the worker and queen samples. (B) Gut microbiota composition of all samples at the phylum level. (C) Gut microbiota composition of all samples at the genus level. (D) Comparative analysis of bacteria genera.

groups were also Proteobacteria, these bees showed more Firmicutes and Actinobacteria than the CK bees; the QG bees had the highest amounts of these bacteria (Fig. 2B). At the genus level, the most obvious differences between QG, WG, and CK bees were that the guts of the QG and WG bees were rich in *Lactobacillus*, *Bifidobacterium*, and *Bombella* bacteria, while CK bees had no *Lactobacillus* or *Bifidobacterium* and only a small amount of *Bombella* bacteria. The guts of CK bees were mainly occupied by a large number of *Klebsiella*, *Bartonella*, and some unclassed miscellaneous bacteria. Statistical analysis of the gut bacteria at the genus level showed that QG bees had a higher abundance of *Lactobacillus*, *Bifidobacterium*, and *Bombella* bacteria than WG bees (Fig. 2D), which was similar to the characteristics of the colony queen bee's gut microbiota. This suggested that the method of gut microbiota transplantation successfully reshaped the gut microbiota structure and mimicked the profiles of the colony queens' and worker bees' gut microbiota.

Gut microbiota from diverse origins induced differences in metabolites involved in lifespan pathways

In general, gut microbiota can interact with hosts via metabolites. Thus, to explore the mechanisms behind the relationship between gut bacteria and lifespan, the intestines of CK, QG, and WG bees were analyzed using untargeted metabolomics to identify potential metabolites that regulate lifespan. Differences in metabolite compositions between groups were tested using orthogonal partial least squares-discriminant analysis (Fig. 3A through F) and displayed using heat maps (Fig. 3G through I). There were significant differences in metabolite compositions of the CK, WG, and QG groups. VIP scores (38) were used to infer biomarkers from the multivariate models (threshold: VIP ≥ 1), and the Student's *t*-test was performed to select them in a univariate fashion ($P < 0.05$) (39). In total, 1,580 (CK vs QG), 1,953 (CK vs WG), and 1,673 (WG vs QG) DMs were identified (Fig. S2).

To explore the functions of the DMs in the different groups, KEGG pathway enrichment analysis was performed for all identified DMs. Sixteen lifespan-relevant pathways, including 49 DMs, were shown to be enriched (Fig. 4A through C; Fig. S3A). Of these, pathways with RF ≥ 0.5 or $P < 0.05$ were considered candidate pathways involved in the regulation of longevity by gut bacteria (Fig. 4D). The IIS pathway refers to a conserved neuroendocrine signaling pathway affecting animal lifespans (4, 5). The inhibition of IIS extending animal lifespans was correlated with the activation of antioxidant genes,

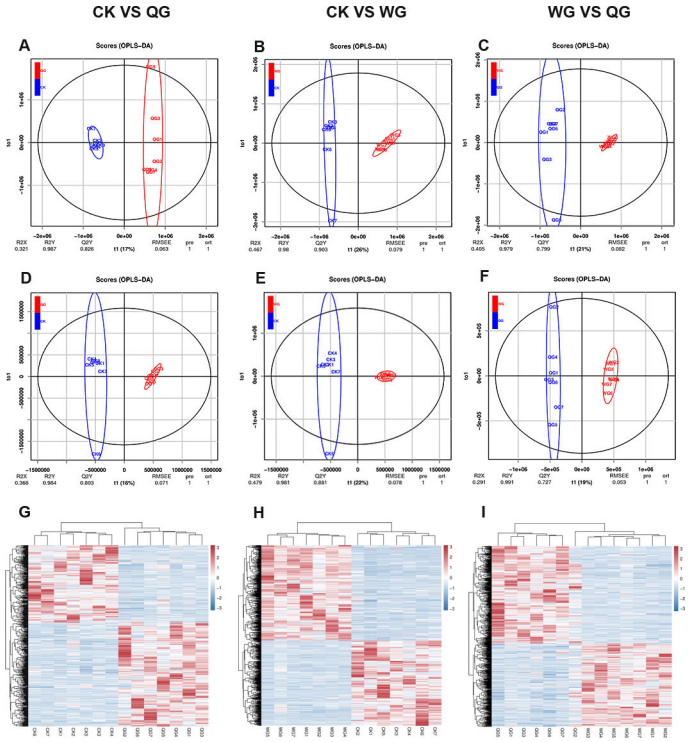

FIG 3 Metabolite compositions in the CK, WG, and QG groups. (A–C) Orthogonal partial least squares-discriminant analysis of metabolites in the positive ion mode. (D–F) Orthogonal partial least squares-discriminant analysis of metabolites in the negative ion mode. (G–I) Global heat map of metabolites.

suggesting a potential mechanism. Inhibiting IIS facilitated the localization of FOXO to the nucleus. The nuclear localization of FOXO, a nuclear transcription factor, increased the transcription of downstream target genes, including antioxidant genes. Antioxidant enzymes could extend lifespan by removing ROS (13). Thus, the candidate pathways of insulin (KO04910), FOXO (KO04068), and glutathione metabolism (KO00480) are likely to be involved in lifespan regulation. The second category of candidate pathways consisted of melanogenesis (KO04916) and intestinal immune networks (KO04672), both of which are immune pathways. Weakened immunity has been identified as a common feature of aging (14). Another candidate pathway was the synthesis and degradation of ketone bodies. The ketone body identified in the comparisons of CK vs QG and WG vs QG was AA (Fig. S3). Thus, the key enzyme for producing AA was subsequently investigated. In general, three categories of potential candidate pathways were identified through which gut microbiota may regulate host lifespans, which included the IIS pathway, immune pathways, and the ketone body metabolism pathway.

QG transplantation bees exhibited IIS expression patterns and antioxidant properties similar to natural queen bees

IIS was investigated in this study to clarify the mechanisms behind lifespan extension after QG transplantation. The IIS activity of queen bees was inhibited compared with that of worker bees in the natural honeybee colony (Fig. 5A). This may be one of the reasons why queen bees live significantly longer than worker bees. Since the mechanisms of insulin signaling, the inhibition of which extends lifespan, are conserved across animals, high levels of nutrition consumption can usually activate IIS. However, what causes the inhibition of IIS in queen bees that consume more nutrition than worker bees remains unknown. The present results showed that IIS was inhibited significantly when QG was transplanted to MF bees compared with the CK and WG groups (Fig. 5B). The expression patterns of ILPs and the insulin receptor (InR) in the QG and WG groups were similar to

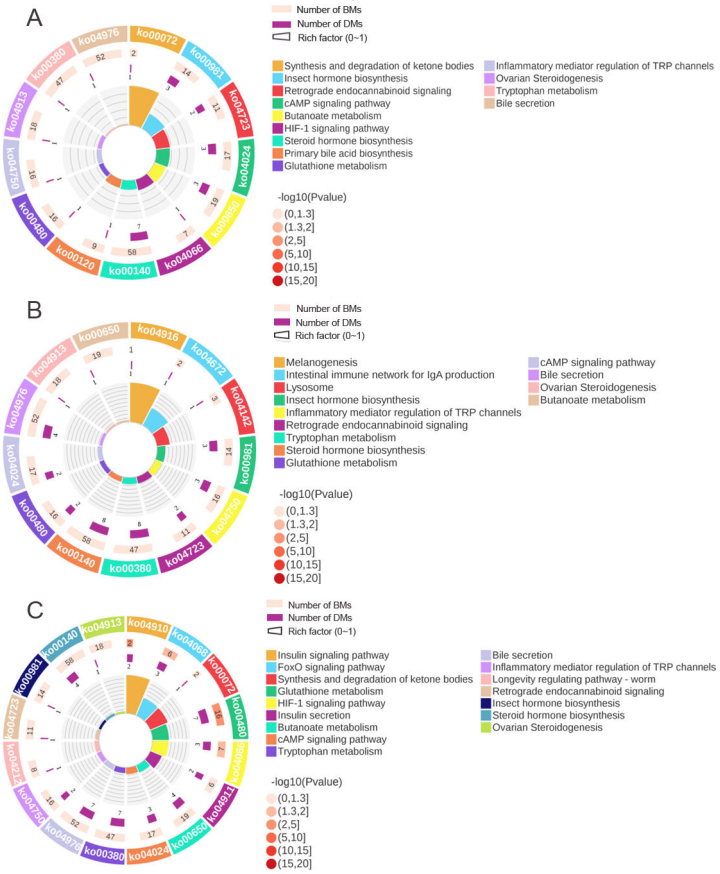

FIG 4 Lifespan-related pathways involving differential metabolites. (A) CK vs QG. (B) CK vs WG. (C) WG vs QG. Outer colorful arcs represent the pathway ID; the number of pink rectangles in the second ring represents the number of background metabolites (BMs); the shade of the pink rectangles in the second ring represents the $-\log_{10}$ *P*-value; the number of purple rectangles in the third ring represents the number of significant differential metabolites (DMs); the fanshape in the innermost ring represents the rich factor (BM number/DM number).

those of natural queen and worker bees. Compared with the CK group, the QG and WG groups showed inhibited expression of ILPs. These results demonstrated that both QG and WG transplantation could inhibit IIS activity, whereas the suppression of IIS by QG transplantation was stronger than that by WG transplantation.

The mechanism of inhibiting IIS extending the animal lifespan was found to be correlated with the activation of antioxidant genes as mentioned above, consistent with our results that the expressions of *catalase*, *SOD1*, *SOD2*, *cytochrome B,* and *cytochrome B* in queen bees were higher than that in worker bees (Fig. 5A). A similar trend reappeared in QG and WG transplantation bees, whereas no statistically significant difference was found (Fig. 5B). Compared with MF bees in the CK group, the expression levels of *catalase*, *GST*, *SOD1*, *SOD2*, *cytochrome B,* and *cytochrome B* were significantly upregulated by QG and WG transplantation ($P < 0.05$). To assess the degree of oxidative damage, malonaldehyde, 4-hydroxynonenal acid, protein carbonyl, and 8-hydroxydeoxyguanosine were detected as the product of lipids, protein, and DNA oxidative damage. Similar to natural queen bees, QG had the lowest MDA, 4-HNE, PC, 8-OHDG, and ROS (Fig. 5C and D). WG transplantation could also reduce the levels of some oxidative damage products compared with MF, but not all. The above results at least revealed that one of the mechanisms by which queen bees live longer than worker bees would be reducing the degree of oxidative damage by upregulating antioxidant genes' expressions via inhibiting the IIS pathway. QG transplantation mimics this process magically. It was suggested

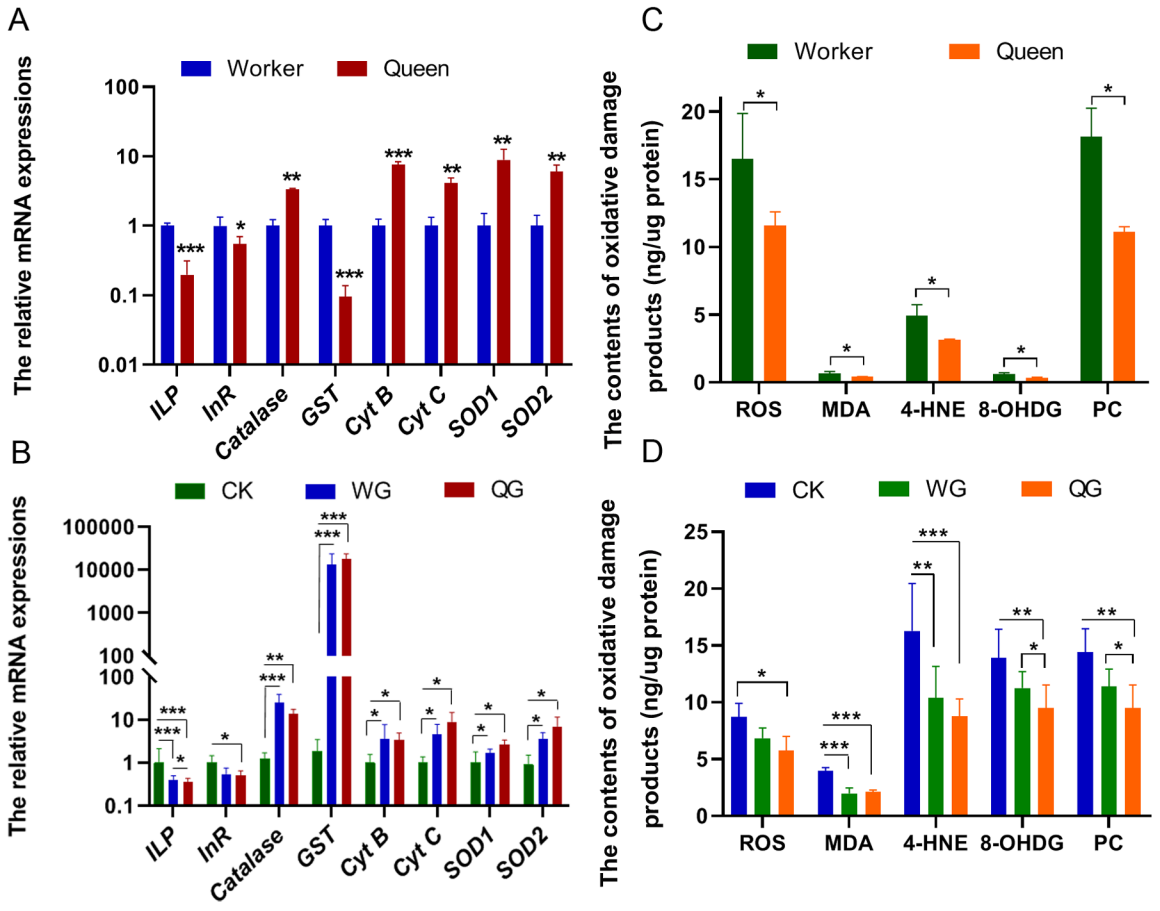

FIG 5 The mRNA expression patterns of IIS, antioxidant enzyme genes, and oxidative damage products. (A) The mRNA expression pattern of IIS and antioxidant enzyme genes of natural queen and worker bees. (B) The mRNA expression pattern of IIS and antioxidant enzyme genes of MF (CK) and WG- and QG-transplanted bees. (C) Oxidative damage products in natural queen and worker bees. (D) Oxidative damage products in MF (CK) and WG- and QG-transplanted bees. MDA, lipid oxidation damage product malonaldehyde; 4-HNE, lipid oxidation damage product 4-hydroxynonenal acid; PC, protein oxidation damage product protein carbonyls; and 8-OHDG, DNA oxidation damage product 8-hydroxydeoxyguanosine.

that QG transplantation might extend the host lifespan at least in part by inhibiting IIS and enhancing antioxidant action.

Gut microbiota transplantation could not completely simulate the immune properties of natural queen and worker bees

Based on the potential candidate pathways from the metabolomics data, the effects arising from gut microbiota transplantation on bee immunity were investigated. The honeybee's immune system consists of cellular immunity and humoral immunity. Hemocyte numbers were analyzed to determine the cellular immunity abilities of the bees. The Imd/relish pathway refers to the classical immune pathway that regulates the synthesis of antimicrobial peptides, representing the humoral immunity of honeybees. Melanization is also a form of humoral immunity. Phenoloxidase, as the core enzyme of melanization (40), was detected in the present analysis. In natural honeybee colonies, despite the lower Imd activity in queen bees, the expression of the downstream transcription factor relish in queen bees was significantly higher than that of worker bees, which promoted the higher expression of downstream target genes related to antimicrobial peptides (*hymenoptaecin*, *defensin*, *abaecin*, and *apidaecin*) ($P < 0.05$) (Fig. 6A). However, the mRNA level of phenoloxidase in queen bees was significantly lower than that in worker bees ($P < 0.05$) (Fig. 6A). In terms of cellular immunity, queen

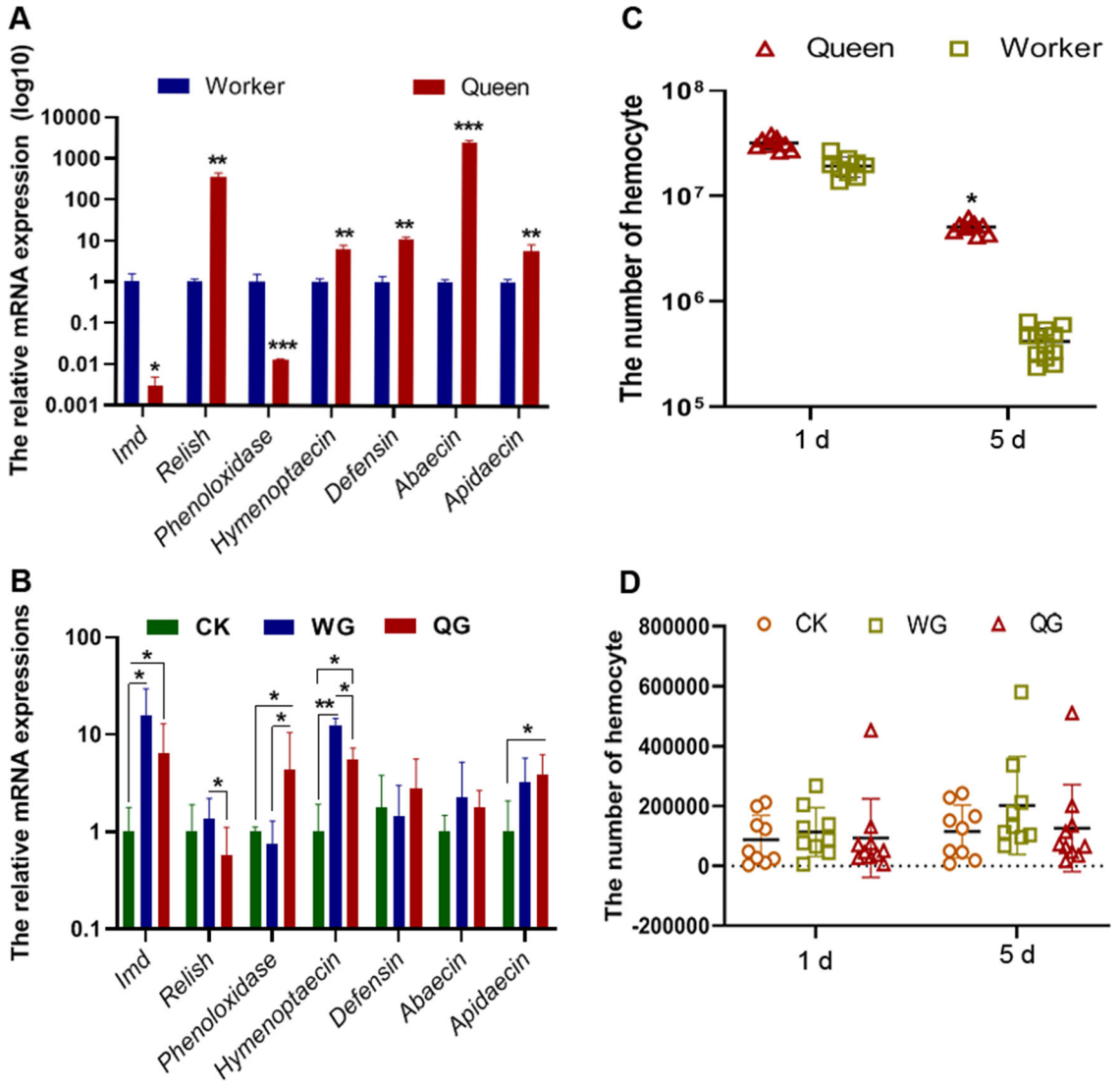

FIG 6 Immune performance. (A) Expression levels of immune-related genes of natural queen and worker bees. (B) Expression levels of immune-related genes of MF (CK) and WG- and QG-transplanted bees. (C) The number of hemocytes of natural queen and worker bees. (D) The number of hemocytes of MF (CK) and WG- and QG-transplanted bees.

bees possessed significantly more total hemocytes than worker bees ((P < 0.05) (Fig. 6C). The number of hemocytes can, to some extent, reflect the cellular immunity capacity (15, 41). These results suggested that the queen bees had more powerful cellular and humoral immunity than worker bees in a natural colony. This supported the viewpoint that weakened immunity is a common feature of aging (14). To some extent, QG or WG transplantation altered humoral immunity; for example, QG and WG transplantation upregulated *Imd* and *hymenoptaecin* mRNA expression (P < 0.05), and QG transplantation upregulated *phenoloxidase* and *apidaecin* mRNA expression (P < 0.05). However, there was little difference in the effects of QG and WG transplantation on humoral immunity, with QG only affecting (downregulating) the expression of *relish* and *hymenoptaecin* compared with WG (P < 0.05) (Fig. 6B). At the same time, neither QG nor WG changed hemocyte numbers. These results suggested that QG and WG transplantation could not completely mimic the immune profiles of natural queen and worker bees. In other words, the increased lifespan after QG transplantation was likely not mediated by immune pathways.

The ketone body metabolism profiles in natural queen and worker bees were different from those in QG- and WG-transplanted bees

The ketone body metabolism pathway was an identified candidate pathway through which gut microbiota may regulate host lifespans. One ketone body that was different among the three groups, AA, was investigated (Fig. S3). 3-hydroxy-3-methyl glutaryl CoA synthase (HMG-S) and 3-hydroxy-3-methyl glutaryl CoA Lyase (HMG-L) are the core enzymes for producing AA (42). The expression profiles of HMG-S and HMG-L in natural queen and worker bees, as well as those in the QG and WG groups, were assayed. The expression levels of HMG-S in natural queens were higher than those in natural workers. However, the expression levels of HMG-S in QG bees were lower than those in WG bees. This suggested that ketone body metabolism in natural queen and worker bees could not be reproduced by transplanting QG or WG to MF bees. AA was the only ketone body that differed among the three groups, and it was most abundant in QG bees (Fig. S3). Thus, the effects of AA on lifespan were tested, and the results were not consistent with expectations. AA shortened the lifespans of bees in a dose-dependent manner (Fig. 7B). This suggested that the ketone body metabolism pathway was not the effective pathway through which QG prolonged the bees' lifespans.

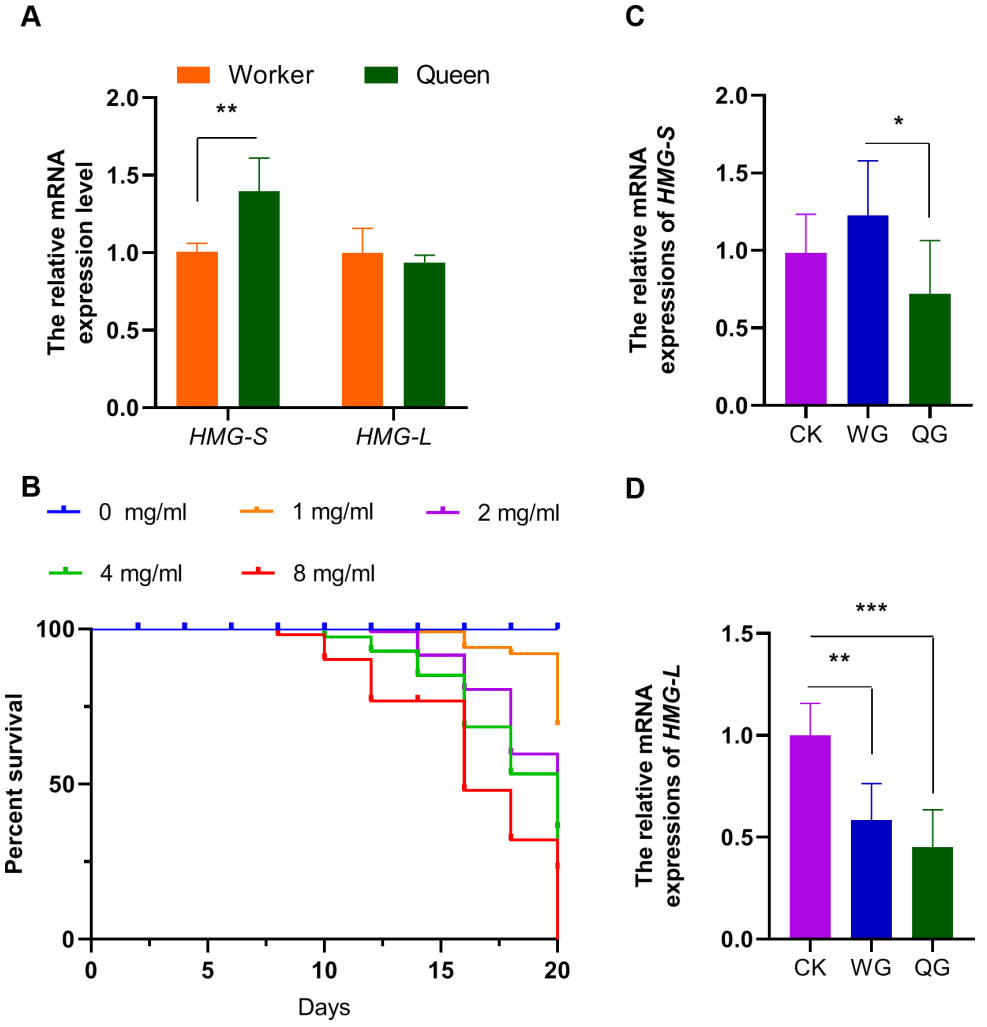

FIG 7 Ketone body metabolism profiles. (A) Expression levels of HMG-S and HMG-L of natural queen and worker bees. (B) Effects of the ketone body acetoacetic acid on bee lifespan. (C) and (D) Expression levels of HMG-S and HMG-L in MF (CK) and WG- and QG-transplanted bees.

DISCUSSION

Herein, we attempted to uncover the effects and mechanisms behind the relationship between queen bee gut microbiota and host bee lifespans using young MF bees transplanted with QG or WG. We showed that transplantation of QG into MF recipient bees extended their lifespan. Earlier studies on the gut microbiota composition of queen and worker bees can shed light on this finding. Queen and worker bees have different dominant gut microbiota; the dominant microbiota in the worker bee's gut largely consists of Proteobacteria (*Gilliamella*, *Frischella*, and *Snodgrassella*), and the dominant microbiota in the queen bee gut primarily includes Acetobacteraceae (Alpha 2.1 and Alpha 2.2) and Firm-4 and Firm-5 *Lactobacillus* (20, 22, 43). The present results showed that QG-transplanted MF bees had more abundant *Lactobacillus*, *Bifidobacterium*, and *Bombella* bacteria than WG-transplanted MF bees and control CK bees; moreover, CK bees had no *Lactobacillus* or *Bifidobacterium* bacteria. One reasonable explanation for this is that *Lactobacillus* and *Bifidobacterium* bacteria, recognized as beneficial gut bacteria, improve health and extend queens' lifespans. However, little is known about the function of *Bombella* (belonging to Alpha 2.2 taxa) bacteria in honeybees. Our previous study found that *Bombella* bacteria were a queen-biased biomarker taxon and suggested that *Bombella* bacteria should be beneficial to honeybees (35). Other studies also suggested that queen bee guts harbor more Acetobacteraceae members (especially Alpha 2.2) than worker bee guts (20, 22, 43). Food is an extremely important factor affecting the differentiation of honeybees into castes. The main food of queen bees is RJ, unlike the worker bee diet of bee bread and honey (44). Feeding RJ to worker bees can prolong their lifespan (7). The mechanisms by which RJ extends lifespan have remained elusive, except for the effects of the antioxidant components of RJ. Previous studies have shown that Acetobacteraceae (Alpha 2.2) bacteria were prolific in some groups (crops, hypopharyngeal glands of nurse bees, RJ, and larva fed on RJ) characterized by their access to RJ (45–47), thus promoting the notion that the queens' unique diet may create their unique gut microbiota, which may contribute to longevity.

As revealed by existing studies, the potential mechanisms behind queen bees living longer than worker bees mostly comprise IIS, immune, and antioxidant pathways. Thus, the question is raised of which pathways are behind the effects of QG on longevity. The present study found that QG transplantation not only extends the longevity of bees but also increases food intake and dry matter deposition. This seems to contradict the finding that dietary restriction tends to increase lifespans in model organisms (48). The fact that dietary restriction can extend life expectancy is true only if organisms are nourished adequately, without malnutrition. The lower food intake of MF bees compared with the WG-transplanted bees (approximately equal to normal worker bees) suggested that the removal of gut bacteria could inhibit appetite or reduce bees' appetite for sugar, which was also supported by a study by Zheng et al. (49). Under conditions of lower food intake (MF bees), lifespan was positively correlated with food intake (Fig. 1D). However, at higher food intake levels (QG and WG bees), the positive correlation between lifespan and food intake was not found (Fig. 1E and F). This information suggests that gut bacteria may regulate longevity via nutrition-related pathways. The IIS pathway has been the classical nutrient-sensing pathway first discovered to affect the aging process (50, 51). Thus, we speculated that QG transplantation may extend lifespan via IIS. The present metabolomics data support this. We identified three categories of potential candidate pathways, including IIS, immune pathways, and ketone body metabolism, through which gut bacteria may regulate bee lifespans. Subsequently, we validated these three signaling pathways. Only IIS activity in QG- and WG-transplanted bees was found to be highly similar to those of natural queen and worker bees. This meant that transplanting QG to MF bees inhibited IIS activity more than transplanting WG to MF bees. The mRNA expression of downstream antioxidant enzyme genes in QG-transplanted bees was higher than that in WG-transplanted bees, just as normal queen bees had higher antioxidant enzyme gene expression levels than normal worker bees. The mechanisms behind the effect of IIS inhibition extending animal lifespans have been elucidated in

other model animals (52–54). Decreased IIS diminishes the phosphorylation of FOXO, thus leading FOXO proteins to remain in the nucleus. Nuclear FOXO proteins facilitate the transcription of antioxidant genes, including *SOD*, *catalase*, *GSH-PX*, and other genes (51, 55). The upregulation of antioxidant enzymes can effectively clear ROS, delay aging, and extend lifespans. The results of the present study demonstrated that queen bee gut bacteria prolonged the lifespan of MF bees potentially through upregulation of the antioxidant capacity mediated by the inhibition of the IIS pathway. However, a previous study on the transplantation of WG to MF bees showed upregulation of ILP expression (49), which is inconsistent with the present results. The reason for this may be related to different tissues and ages. In the present study, intact honeybees were used for detecting IIS gene expression and were sampled at 9 days of age. In the previous study, the expression levels of ILP genes were examined only in the heads of 7-day-old bees, and InR genes were examined in the abdomens of the same bees (49).

We aimed to gain deeper insight into how gut bacteria transplantation can inhibit IIS. In the present results, gut bacteria transplantation (QG or WG) significantly increased food intake. Theoretically, higher food intake would upregulate IIS, whereas IIS was inhibited after QG and WG transplantation. This was also seen in natural queen bees with higher dietary levels than worker bees having lower IIS activity (10). Therefore, it can be speculated that intestinal bacteria may change the responses of the host IIS to dietary sugar levels. In general, intestinal symbiotic bacteria interact with the host through metabolites, and we attempted to identify the metabolites that could effectively inhibit IIS or regulate lifespan using metabolomics. The differences in lifespan were mainly seen in the comparisons of CK vs QG and WG vs QG, and the DMs enriched in lifespan-related pathways and overlapping in the CK vs QG and WG vs QG comparisons were selected to assess the exact effects on lifespan (Fig. S3 and S4). As revealed by the results, AA, L-lactic acid, and pyroglutamic acid all exerted negative effects on longevity in a dose-dependent manner. Unfortunately, we have not yet identified any metabolites that could extend the lifespan of bees. Transcriptomic and proteomic changes in the aging process revealed several conserved pro-longevity processes, consisting of numerous metabolic changes [e.g., carbohydrate metabolism (56), lipid and fatty acid metabolism (57–59), energy metabolism (56), and protein and methionine metabolism (60)]. In other words, the metabolites involved in the regulation of lifespan are likely a complex system rather than simple contributions of one or a few metabolites. The regulation of longevity by honeybee gut bacteria is highly complex and fascinating, and this should be further studied in depth. KEGG enrichment analysis of metabolites led us to focus on numerous targeted pathways in which hundreds of metabolites remain to be validated (Fig. S5). Further studies should focus on solving this problem.

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All authors have seen and agreed with the contents of the manuscript and there is no conflict of interest, including specific financial interest and relationships and affiliations relevant to the subject of the manuscript.

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AUTHOR CONTRIBUTIONS

Hongfang Wang, Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review and editing | Wenfeng Chen, Methodology, Project administration | Li Lei, Investigation, Methodology, Project administration | Wei Zhang, Project administration | Zhenguo Liu, Software | Ying Wang, Writing – review and editing | Baohua Xu, Funding acquisition, Supervision, Writing – review and editing

DATA AVAILABILITY

All raw data have been deposited in the SRA database (NCBI accession number PRJNA1061371).

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Figures S1-S5 (AEM01799-23-S0001.docx). Supplemental figures.

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