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Nano- and micro-polystyrene plastics disturb gut microbiota and intestinal immune system in honeybee

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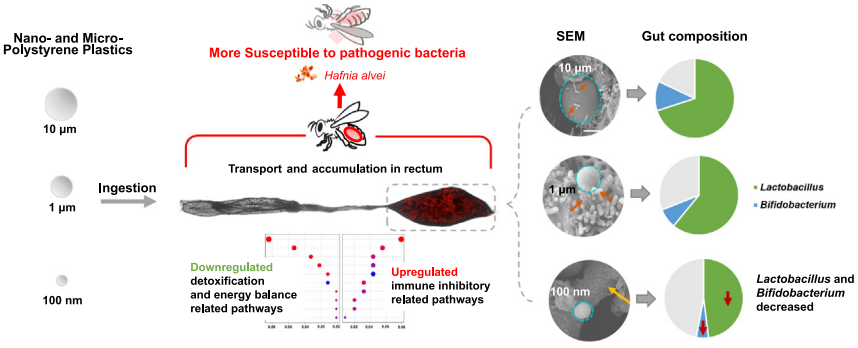
HIGHLIGHTS

- NPs decreased the abundance of *Lactobacillus* and *Bifidobacterium* in bee microbiota.
- NPs stimulated immune inhibitory genes of honeybee.
- NPs depressed genes related to detoxification and energy balance.
- NPs-exposure made bees more susceptible to the pathogenic bacteria *Hafnia alvei*.

GRAPHICAL ABSTRACT



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ABSTRACT

Micro- (MPs) and nano-plastics (NPs) have become emerging pollutants in the environment. Their wide distribution and capacity as a vector of hazardous materials threaten various organisms. Honeybees have been used as bioindicators for pollutants as their gut microbiota offers advantages for addressing how it alters the host health and exploring the processes of environmental pollutants affecting gut community dynamics. In this study, the effects of plastic particles of different sizes on honeybees' health were investigated. Oral exposure to polystyrene (PS) particles with a diameter of 100 nm significantly decreased the whole-body weight and survival rate of honeybees and induced intestinal dysplasia. As the increase of the feeding time from Day 0 to Day 15, the MPs moved to and accumulated in the rectum, where most bee gut symbionts colonized. Scanning electron microscope observation showed that 100-nm PS particles adhered to the germination pore of pollen, while 1- and 10-μm PS particles were attached by gut bacteria. We found that 100-nm PS treatment decreased the relative abundance of *Lactobacillus* and *Bifidobacterium* in the guts. Correspondingly, PS treatment stimulated immune inhibitory genes and depressed genes related to detoxification and energy balance. Furthermore, 100-nm PS treated honeybees became more susceptible to the pathogenic *Hafnia alvei*, leading to a five-times higher mortality rate. These results indicated the adverse impacts of NPs on honeybees, which extends our knowledge regarding the emerging health risks of plastic debris, especially at the nanoscale.

1. Introduction

Microplastics (MPs), defined as plastic fragments smaller than 5 mm, are currently listed as the second most important scientific problem in environmental and ecological science (Amaral-Zettler et al., 2016) because of their wide distribution in aquatic and terrestrial ecosystems (Colton et al., 1974; Cózar et al., 2014; Wang et al., 2019). With small particle

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size and low biodegradability, MPs can exist in the digestion system of living organisms after being ingested and accumulate to a significant level. They subsequently migrate into the food chains and pose considerable health risks to various living organisms and humans (Cole et al., 2013; Mattsson et al., 2015; Torre et al., 2014). Previous reports have shown that ingestion of MPs has complicated negative impacts on animals, including disturbance in gut microbiota (Jin et al., 2019), gene expression dysregulation (Fadare et al., 2020), behavioral and physiological dysfunction (Lovern et al., 2007), immunotoxicity (Rochman et al., 2013), and reproductive defects (Torre et al., 2014).

The effects of MPs with different sizes on organisms are markedly diverse (Lee et al., 2013). The accumulation and distribution of MPs strongly correlate with their size (Wright et al., 2013). It was reported that MPs of 5 and 70 nm were identified in zebrafish's liver, whereas particles of 20 μm were absent, indicating MPs with different particle sizes may have different effects on organisms (Lu et al., 2016). Fragmentation of plastics occurs over time in nature, leading to a continuous decrease in the average size of plastic particles in the environment over the years (Cai et al., 2021). Thus, nanoplastics (NPs, <1000 nm) are gaining increasing attention worldwide as an emerging hazardous material in the environment (Rocha-Santos and Duarte, 2014). Compared to MPs, the smaller particle sizes of NPs make them easier to be ingested by living organisms and, therefore, may pose higher ecological and health risks (Cai et al., 2021). Additionally, such small fragments pose another health threat from the chemical pollutants readily absorbed by NPs due to their high surface reactivity (Rochman et al., 2013).

Honeybees (*Apis mellifera*) or their products can serve as excellent bioindicators for environmental pollutants. Each colony would have several thousands of worker bees that usually cover kilometers in range during their foraging activity (Devillers and Phamdelegue, 2002). When honeybees gather pollen, they will also act as unintentional samplers of environmental contaminants, eventually accumulating in bee products such as honey, propolis, royal jelly, and wax (Żaneta et al., 2016). MPs in honeybees from apiaries near semi-urban and rural areas have already been reported (Edo et al., 2021). A recent study found that 50-nm PS damaged the midgut tissue of honeybees, resulting in the transfer of PS into the trachea, hemolymph, and Malpighian tubules. Genes related to immune response, the respiratory system, membrane lipid metabolism, and detoxification were also affected (Deng et al., 2021).

The gut microbiota is of particular interest because of its various impacts on host health. It can improve food digestion, bolster the immune system, and increase resistance against pathogens (Kamada et al., 2013; Rooks and Garrett, 2016; Trompette et al., 2014). The gut microbiota of the honeybee is an excellent experimental model for studying the relationship between gut microbiota and hosts. It has been used to investigate the dynamic changes in the composition and diversity of the gut community. Compared to mammals (Wang et al., 2018), honeybee gut microbiota's simplicity and conservative nature make them the ideal model system, especially when studying the effect of external stress, such as MPs or pathogenic bacteria. A previous study showed that polystyrene (PS) MPs had a limited impact on the whole body weight and mortality of bees. However, it significantly altered the gut microbiota structure and the expression of genes associated with the immune system, antioxidative, and detoxification (Wang et al., 2020). However, only MPs with a diameter of 25 μm were studied, and the effects of nano-scaled particles on honeybees remain elusive. Hence, further studies of the impact of both N/MPs with different sizes on the honeybee and its gut microbiota are needed.

This study aims to assess the influence of different particle sizes of PS N/MPs on honeybees, especially at the nanoscale, and the susceptibility of honeybees to pathogenic bacteria after exposure to PS N/MPs. Such impact on the survivorship and whole-body weight change of the honeybees was investigated. Then, the accumulation of MPs and the gut development were observed simultaneously. We explored gut microbiota and gene transcription alteration in honeybees after N/MPs exposure. Finally, the changes in honeybees' resistance to the pathogenic bacteria post-exposure to NPs were analyzed. Our results will provide a better understanding of the potential health risks associated with the emerging environment-concerning plastic debris, especially at the nanoscale.

2. Material and method

2.1. N/MPs preparation

The pristine polystyrene MPs with a diameter of 100 nm, 1 μm, and 10 μm, as well as the fluorescence-labeled polystyrene MPs (10 μm, labeled with Nile blue, shown in Fig. S1 in the Supplementary Information), were purchased from Xi'an Ruixi Biotechnology Co. LTD (Xi'an, China). The concentrations of pristine polystyrene MPs and fluorescence-labeled polystyrene MPs are 25 and 10 mg/ml, respectively. According to the previous study, the soil samples in the industrial area contain 0.3×10^3 to 67×10^3 μg/ml of microplastics (Fuller and Gautam, 2016). We dilute the N/MPs to 10^5 and 10^4 particles/ml, achieving the final concentration of 10^{-7}– 10^3 μg/ml to mimic the concentrations of microplastics found in the industrial area.

2.2. Animal experiments

Honeybees (*Apis mellifera*) used in this study were collected from our apiary at Li Bao Road, Shunyi, Beijing, China, and they are all 10-day-old worker bees from a single colony. Ten individual bees were kept in a customized cup cage with holes made for ventilation in the dark incubator at 35 ± 1 °C and 50 % relative humidity for 15 days. After starvation for 4 h, honeybees were provided with selected diets of 0.5 g pollen and 1.5 ml sucrose syrup. For the control group, a regular diet consists of pollen grains and 50 % sucrose syrup. For the exposure to N/MPs of different sizes, a regular diet containing 10^5 particles/ml of 100-nm, 1-μm, and 10-μm PS was provided. For the exposure to NP of different concentrations, diets of pollen and 50 % sucrose syrup containing 10^4 and 10^5 particles/ml of 100-nm PS were provided. For fluorescence-labeled MPs exposure, a diet of pollen and 50 % sucrose syrup containing 10^5 particles/ml of 10-μm PS labeled with Neil blue was provided. The diet supply was monitored daily, and when the microplastic-spiked sucrose syrup was depleted, sucrose syrup without microplastics was replenished to ensure a sufficient food supply. During 15 days of incubation, the number of living individuals in the cages was counted every day. The whole-body weight of honeybees was recorded on Day 0, 3, 6, 9, 12, and 15. Each honeybee was measured with an electric balance (CP522C, Beijing, China) immediately after being immobilized at 4 °C. The whole guts of the honeybees from different treatment groups were dissected on Day 15 using fine-tipped forceps as previously described (Zheng et al., 2017). The whole guts of the honeybees from the fluorescence-labeled MPs exposure group were extracted at 2 h, 12 h, 1 d, 2 d, 3 d, and 15 d using the method above.

2.3. Histopathological analysis and microscope observation

The colon extracted from the honeybee was fixed immediately in formaldehyde solution (10 % v/v). Subsequently, the fixed tissues were treated in a series of ethanol-water with ethanol concentrations at 75 %, 85 %, 95 %, and 100 % to dehydration, followed by hyalinization in xylene and embedment using paraffin wax at 56 °C. A microtome (RM2235, Leica camera, German) was used to cut the tissues into specimens with 5 μm thickness before they were stained using hematoxylin-eosin (H&E) solution. The intestinal wall thickness and crypt depth were determined using Image-Pro Plus 6.0 software (Media Cybernetics, Georgia, USA).

Bee guts containing fluorescence-labeled MPs were observed by an inverted fluorescence microscope (TI-S, NIKON, Japan). The fluorescence-labeled PS was detected using the blue excitation filter block. For SEM observation, the guts of honeybees were punctured to obtain the inside homogenate with 4 % paraformaldehyde fixative, and the homogenate was soaked for 12 h and centrifuged at 7500 r/min for 10 min. The resulting supernatant was removed and added with 30 % ethanol (diluted with PBS). Then, it was shaken and stood for 10 min and then centrifuged for

5 min (7500 r/min). Serial concentrations of ethanol were applied, and the final precipitation was lyophilized using a lyophilizer (LGJ-18, Beijing Songyuan Huaxing Technology Development Co., Ltd., Beijing, China). The final powder was put on the conductive adhesive and observed using an SEM system (SM-7401F, JEOL Company, Japan).

2.4. High-throughput 16S rRNA sequencing of gut microbiota

FastDNA® Spin Kit (MP Biomedicals, USA) was used to extract DNA from guts. The DNA concentration and purity were determined using NanoDrop 2000 (Thermo Scientific, Wilmington, USA). The V3-V4 region of the bacterial 16S rRNA gene was amplified (primer set 338F/806R). The PCR amplification was conducted according to a published protocol (Chen et al., 2021). The AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) was used to purify the PCR products. The Quantus™ Fluorometer (Promega, USA) was used to quantify PCR product. Purified amplicons were mixed in equivalent proportions. Sequencing was performed by the Illumina MiSeq PE300 platform (Illumina, USA). Fastp version 0.20.0 was used to perform quality filtering of the raw 16S rRNA reads (Chen et al., 2018), and FLASH version 1.2.7 was used to merge them (Magoč and Salzberg, 2011). UPARSE version 7.1 was used to cluster operational taxonomic units (OTUs) with a 97 % similarity threshold (Edgar, 2013), and chimeric sequences were deleted. RDP Classifier version 2.2 was used to analyze the taxonomy of OTU sequence against the 16S rRNA database (Silva v138) with a threshold of 0.7 (Wang et al., 2007). The analysis is performed on the Majorbio Cloud Platform.

2.5. RNA extraction, library construction, and transcriptome sequencing of bee guts

The methods here were referred to a previous manuscript with modifications (Zhang et al., 2022). Total RNA of bee guts was obtained using the Quick-RNA MiniPrep kit (Zymo Research, USA). Bioanalyzer 2100 system (Agilent Technologies, USA) was used to assess RNA integrity. The NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs, USA) was used for RNA library construction and added index codes for samples. The sample cluster was performed on cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS. The library preparations were sequenced on an Illumina NovaSeq 6000 platform (Illumina, USA) with a high-output 150 bp sequencing run.

FastQC version 0.11.5 was used to assess sequencing quality. HISAT2 version 2.0.5 was used to build the index of bee reference genome (Amel_HA version 3.1), and HISAT2 version 2.1.0 was used to align the FastQC trimmed reads. HTSeq version 0.7.2 was used to quantify the gene expression. EggNOG-mapper version 5.0 was used to get a better annotation of the bee reference genome. DESeq2 and clusterProfile packages in R were used to obtain differential expressed genes and functional analysis. The gene with p-value < 0.05 and fold change > 2 was assigned as differentially expressed. Functional analysis of differentially expressed genes was performed based on KEGG Orthologue (KO) markers. The percentages of KO markers belong to each category (KEGG Class at level 3).

2.6. The susceptibility of honeybees to H. alvei after exposure to NPs

The sources and feeding conditions of honeybees were the same as described above, and the diets for each group are illustrated in Fig. 7A. For honeybees in the control group, the normal diet of pollen and 50 % sucrose syrup was provided. For honeybees in the PS-100 nm group, the normal diet containing 10^5 particles/ml of 100-nm PS was fed for the first 3 days, and then a normal diet was fed for the rest of the experiment. For honeybees from the *H. alvei* group, a normal diet was fed for the first 3 days, and then 50 % sucrose syrup spiked with 1 ml of pre-cultured *H. alvei* (final OD_{600} of mixture = 1.0) was fed for the rest of the experiment. For honeybees from the 100 nm-PS + *H. alvei* group, a normal diet containing 10^5 particles/mL of 100-nm PS was fed for the first 3 days, and then 50 % sucrose syrup was spiked with 1 ml of pre-cultured *H. alvei* (OD_{600} = 1.0) were fed for the rest of the experiment. The honeybees from different treatment groups were dissected and analyzed on Day 10 using the same procedure as described above.

In addition, the hemolymph was extracted by making a small incision above the median ocellus of honeybees, and 10 μl of hemolymph from each live honeybee was gathered using a pipettor according to a previous method (Zhang et al., 2020). Then, DNA was extracted from hemolymph samples. PCR amplification was performed to identify the bacterial colonies found in the hemolymph samples with the following thermal cycles: 10 min at 95 °C for one cycle, 30 s at 94 °C for thirty cycles, 30 s at 55 °C, 1.5 min at 72 °C, and finally 10 min at 72 °C. The sequencing was performed by Beijing Hooseen Biology Co., Ltd. with the same primers used in Section 2.4, and gene sequence alignment is performed on the https://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.7. Statistic analysis

Statistical analysis was conducted using R 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria), and results were reported as mean ± standard deviation (SD). For whole-body weight change analysis, the Mauchly method was used, and then MANOVA was conducted. The log-rank test was performed on the Kaplan-Meier survival curve. For intestinal wall thickness, crypt depth/intestinal wall thickness ratio, and gut microbiota analysis, the Kruskal-Wallis rank-sum test was conducted, followed by the Game-Howell test. Paired *t*-test or Wilcoxon matched-pairs test was conducted to compare the gut microbiota and predicted KEGG pathways between different groups. The alpha diversity (the Chao1, ACE, Shannon, and Simpson estimator) and PCoA with unweighted Unifrac Bray-Curtis distance were shown using R 4.0.3 (Inc., MA, USA).

3. Results

3.1. Effects of N/MPs exposure on survivorship and whole-body weight of honeybees

To investigate the effect of particle size of PS N/MPs on the growth of honeybees, we fed bees N/MPs with a diameter of 100 nm, 1 μm, and 10 μm at the concentration of 10^5 particles/ml, denoted as PS-100 nm, PS-1 μm, and PS-10 μm. As shown in Fig. 1A, during 15 days of treatment, the natural mortality of the honeybees is around 2 %. Although all treatment groups showed higher mortality rates after 9 days than the control group, such discrepancy in the survivorship is not statistically significant. The whole-body weight of honeybees exhibited the same trend among all treatment groups, where it increased for the first 9 days of incubation and then decreased slightly, whereas the reduction of the body weight occurred after 12 days in the control group (Fig. 1B). On Day 15, a significant difference in whole-body weight of the bees among the 4 groups was observed, and exposure to 100-nm PS led to a significant decrease to 91.67 % of the original weight. In addition to the size of the N/MPs, live organisms have diverse responses to different concentrations of N/MPs (Opitz et al., 2021; Ziajahromi et al., 2018). We then studied the effects of different concentrations of NPs on honeybees. Honeybees were fed with 100-nm PS at 10^4 and 10^5 particles/ml, denoted as PSL and PSH groups, respectively. No significant differences were found among the survivorship of the three tested groups, but the whole-body weight of bees in the PSH group was significantly lower after 9 days (Fig. 1C and D). Our results showed that 100-nm PS at 10^5 particles/ml is an appropriate dose that causes weight change in honeybees without killing them over this short period.

3.2. Effects of N/MPs exposure on gut development of honeybees

The midgut of bees is the primary site for digesting food and absorbing nutrients; hence, a well-developed midgut plays an essential role in digestion and absorption (Santos et al., 2016). To explore the impact of PS N/MPs on gut development, we examined the morphology of midguts

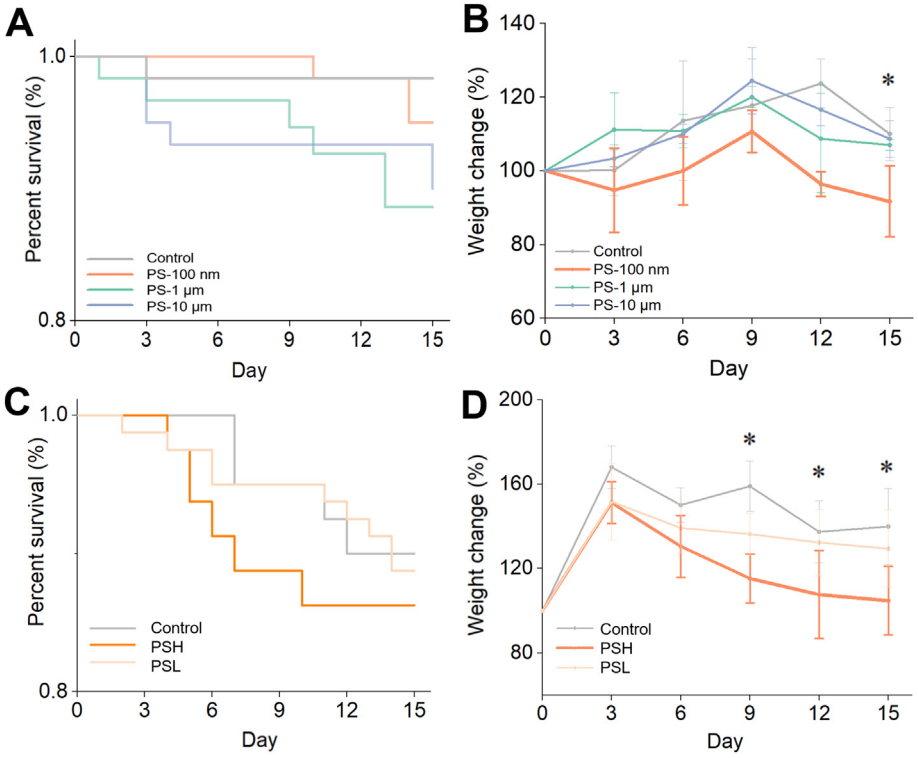

Fig. 1. Survivorship and whole-body weight change of honeybees after N/MPs exposure. (A) Kaplan-Meier survival curve of honeybees' exposure to N/MPs with the diameter of 100 nm, 1 μm, and 10 μm at 10^5 particles/ml (denoted as PS-100 nm, PS-1 μm, and PS-10 μm). (B) The whole-body weight change of honeybees' exposure to N/MPs with a diameter of 100 nm, 1 μm, and 10 μm at 10^5 particles/ml. (C) Kaplan-Meier survival curve of honeybees' exposure to PS with a diameter of 100 nm at 10^5 and 10^4 particles/mL (denoted as PSH and PSL). (D) The whole-body weight change of honeybees' exposure to N/MPs with a diameter of 100 nm at 10^5 and 10^4 particles/ml. Eight replicates were performed for each group. Kaplan-Meier survival model with Log-rank test was used for (A) and (C). Mauchly method was used first, and then MANOVA was conducted for (B) and (D). *P<0.05, **P<0.01, ***P<0.001.

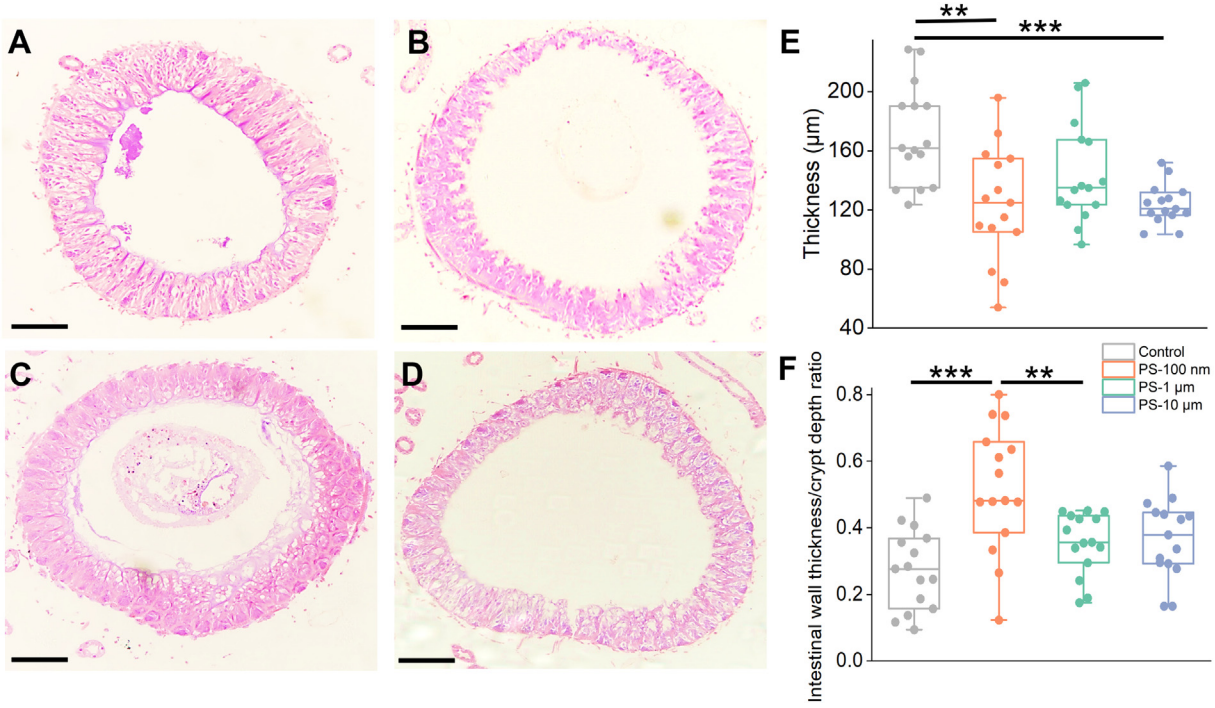

Fig. 2. H&E staining of guts of honeybees from (A) control, (B) PS-100 nm, (C) PS-1 μm, and (D) PS-10 μm groups on Day 15 after initial ingestion. Boxplots of intestinal wall thickness (E) and crypt depth/intestinal wall thickness ratio (F) of honeybees from different treatment groups. Kruskal-Wallis rank sum test followed by Game-Howell was conducted for (E) and (F). The length of the scale label is 100 μm. *P<0.05, **P<0.01, ***P<0.001.

after 15 days of N/MPs exposure by H&E staining (Fig. 2A–D). As shown in Fig. 2A, epithelial cells of guts from honeybees in the control group possess normal nuclei with high stainability and homogenous cytoplasm inclusion with intact cell boundaries. The most abundant type of epithelial cells was columnar cells that were arranged in one layer and settled on the basement membrane. Adverse effects such as raptures of the basement membrane were noted after NPs treatment. The cytoplasm appeared to be vacuolized and lysed with no visible nuclei, as shown in Fig. 2B. The aforementioned adverse effects were alleviated after the MP treatment. In Fig. 2C–D, the loss of matrix was observed in the columnar cells; the basement membrane appeared degenerated with multiple small vacuoles in sight.

The intestinal wall thickness and crypt depth/intestinal wall thickness ratio of honeybees fed with different diets were illustrated in Fig. 2E–F. The honeybees from the PS-100 nm group showed a significantly thinner intestinal wall compared with the control group. Considering that the intestinal wall thickness of honeybees varies greatly, the crypt depth/intestinal wall thickness ratio was used to judge bees' digestive and absorption capacity. A significantly higher crypt depth/intestinal wall thickness ratio value was observed in the PS-100 nm group compared to the control and PS-1 μm group, indicating that the guts of honeybees in the PS-100 nm group were stunted.

3.3. Transport and accumulation of MPs in honeybee guts

MPs often accumulate in the guts after being ingested by organisms, leading to severe adverse effects (Jin et al., 2019). Thus understanding the transport and accumulation of N/MPs in the guts can help identify the possible effects of N/MPs on honeybees. The size of NPs is too small to be observed under a fluorescence microscope, so we chose 10-μm PS for observation. The honeybees were exposed to 10-μm PS at 10^5 particles/ml. MPs first appeared in the midgut of the honeybees at 2 h after initial ingestion and then gradually transferred into the ileum at 6 h (Fig. 3). Then, a portion of MPs reached the rectum at 24 h while most MPs were still in the midgut. After 3 days, MPs were observed mainly in the rectum of bees until Day 15, whereas hardly any MPs were left in the midgut or ileum. In general, the MPs were accumulated at the rectum. Since the rectum harbors abundant gut microbiota (Martinson et al., 2012; Ricigliano and Anderson, 2020), such accumulation of MPs may affect gut microbiota structure there.

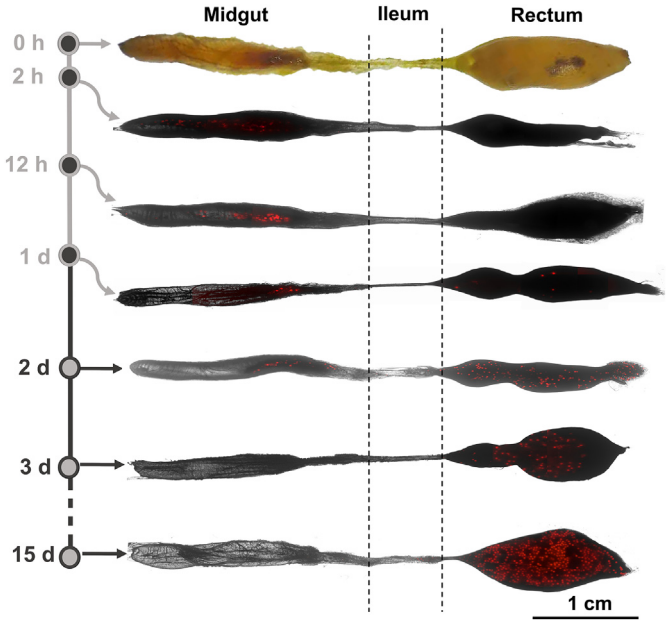

Fig. 3. Transport and accumulation of fluorescence-labeled MPs, with a diameter of 10 μm at 10^5 particles/ml, in the guts of bees during 15 days after initial ingestion.

To further investigate the effects of N/MPs exposure on gut microbiota, we first analyzed the distribution and interaction between N/MPs and intestinal contents by SEM. Pollen with different sizes in the gut homogenate of honeybees from the control group could be observed (Fig. 4A). Germination pores on pollen surfaces are about 0.5–1 μm in width, in which N/MPs could be embedded. Interestingly, the 100-nm PS were adhered to the pollen cell walls and attached to the germination pores of the pollen grains (Fig. 4B). With the particle sizes increasing, MPs had a larger surface than NPs covered by gut bacteria. The 1-μm PS and the gut microbiota are approximately the same sizes, and gut bacteria aggregated around the PS particles (Fig. 4C). In contrast, the size of 10-μm PS is substantially larger than bacterial cells, resulting in more gut bacteria surrounding and attaching to the surface (Fig. 4D). These results suggest that NPs could be embedded in the pollens, whereas the surface of MPs would serve as a habitat for gut microbiota growth.

3.4. Effects of N/MPs exposure on honeybee's gut microbiota composition

To reveal the gut microbiota profile of honeybees after N/MPs exposure, 16S rRNA analysis of gut microbiota was performed on the control, PS-100 nm, PS-1 μm, and PS-10 μm groups on Day 0, 3, 5, 10, and 15 after initial ingestion. No significant difference in alpha diversity was found in either Chao1, Shannon, or Simpson indices (Fig. 5A–C). It might be due to the relatively conservative gut microbiota in honeybees, with only 5–9 species being the dominant species (Zheng et al., 2018). The initial relative abundance of *Lactobacillus* and *Bifidobacterium* of honeybees on Day 0 was 72 % and 12.33 %, respectively (Fig. 5F). However, the relative abundance of *Lactobacillus* of honeybees from the PS-100 nm group significantly reduced to 54.34 % on Day 10 (Fig. 5C), and the relative abundance of *Bifidobacterium* of honeybees from the PS-100 nm group significantly reduced to 6.35 % on Day 15 after the initial ingestion (Fig. 5D).

3.5. Effects of NPs exposure on the transcriptomic profiles of bee guts

To further understand the signal pathways that might contribute to the results we obtained, transcriptional profiles of the honeybees' gut epithelial cells were examined. Since that PS with a diameter of 100 nm at 10^5 particles/ml has the most pronounced effects on host physiology and the gut composition, we compared the transcriptional profiles of control and PS-100 nm groups on Day 15. There were 705 differentially expressed genes (DEGs), and 385 DEGs were up-regulated while 320 DEGs were down-regulated in PS-treated bees from the PS-100 nm group. Notably, several KEGG pathways were substantially different in the honeybee as the result of NPs in their diets. The expression of inflammation-related genes was promoted in the PS-100 nm group, including protein phosphatases and associated proteins, MAPK signaling pathway, ion channels, Rap1 signaling pathway, and thyroid hormone signaling pathway (Fig. 6). Conversely, the expression of genes associated with detoxification, appetite, and glucose & lipid metabolism were down-regulated in the PS-100 nm group, including cytochrome P450, AMPK signaling pathway, and PPAR signaling pathway (Fig. 6).

3.6. Effects of exposure to NPs on the susceptibility of honeybees to pathogenic *H. alvei*

Previous studies showed accumulation of MPs increases susceptibility to pathogenic bacteria in coral reefs (Lamb et al., 2018) and fish (Viršek et al., 2017). To explore honeybees' resistance to potential pathogenic bacteria after 100-nm PS exposure for 3 days, we examined their susceptibility to *H. alvei* (Fig. 7A). The mortality of honeybees from the "*H. alvei*" group and the "PS-100 nm + *H. alvei*" group was significantly elevated compared to those from the "Control" and the "PS-100 nm" groups (Fig. 7B). In particular, mortality of 92 % was observed in honeybees from the "PS-100 nm + *H. alvei*" group, which was 5 and 1.5 times higher

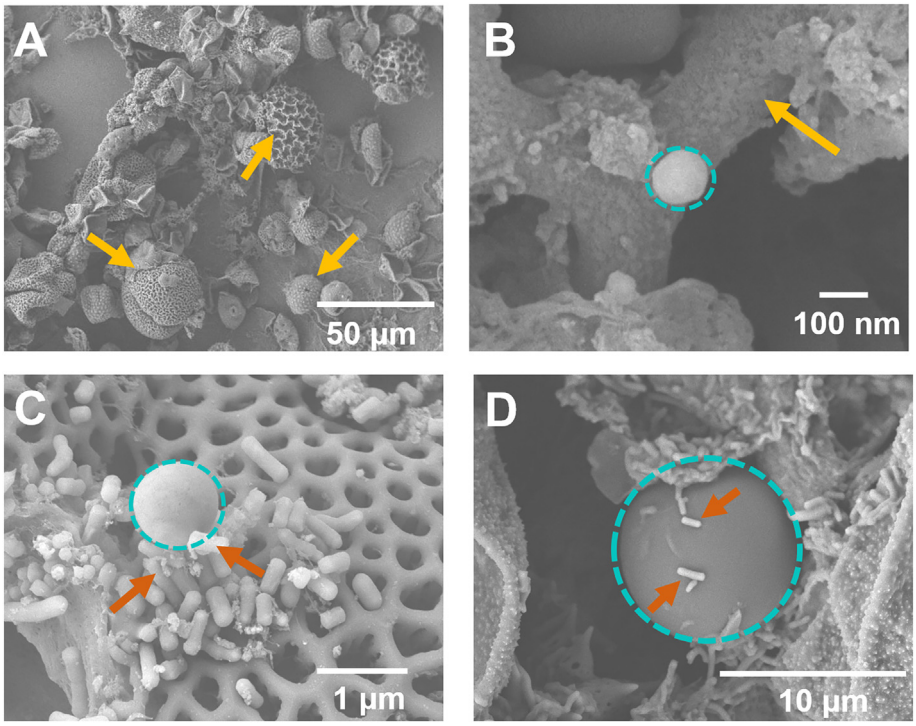

Fig. 4. Scanning electron microscope images of gut homogenate with MPs entering into the midgut of honey bees in (A) control, (B) PS-100 nm, (C) PS-1 μm, and (D) PS-10 μm groups on Day 15 after initial ingestion. Colored arrows point to pollens (yellow) and gut microbiota (black). Blue circles with dotted lines frame the N/MPs.

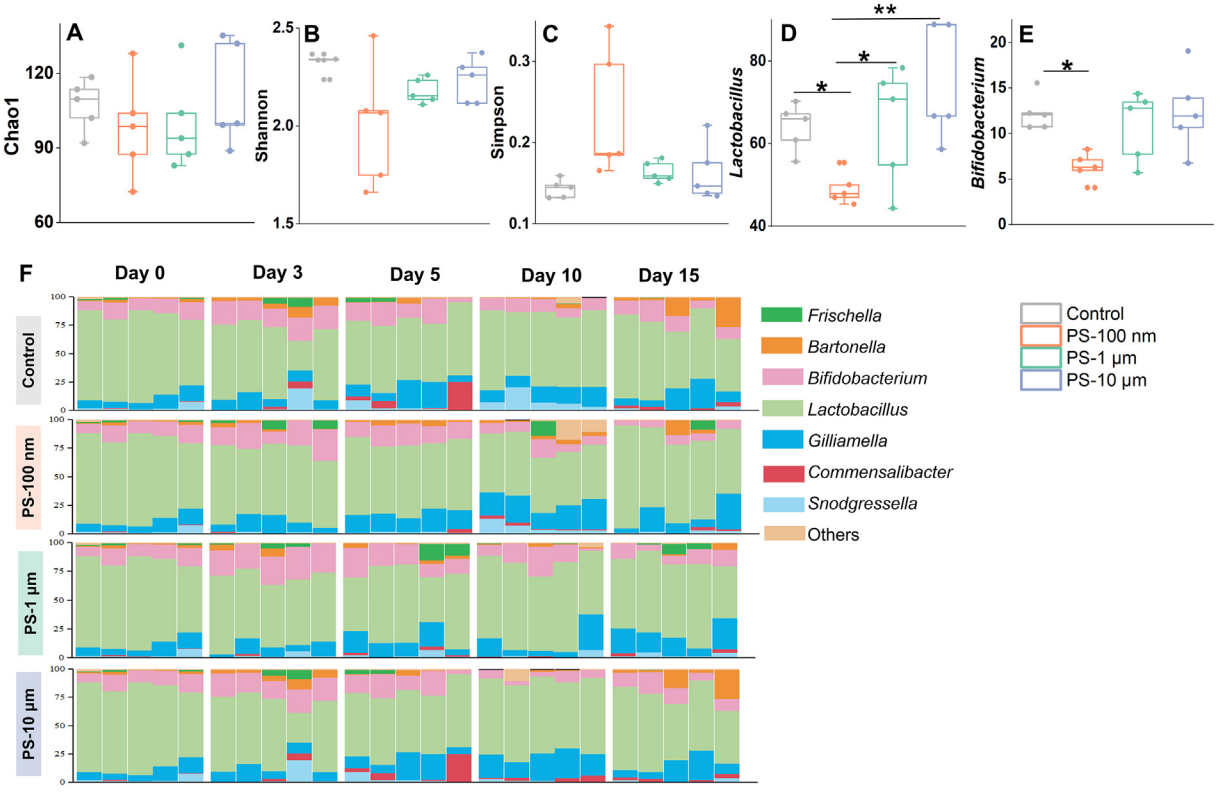

Fig. 5. Changes of gut microbiota structure of honeybees on Day 0, 3, 5, and 15 after initial ingestion. (A) Chao1, (B) Shannon and (C) Simpson indices of gut microbiota of honey bees on Day 15 after initial ingestion. Each column represents one replicate. Relative abundance of (D) *Lactobacillus* and (E) *Bifidobacterium* of honeybees from different treatments on Day 10 and 15 after initial ingestion, respectively. (F) The relative abundances of gut microbiota of honeybees from control, PS-100 nm, PS-1 μm, and PS-10 μm groups on Day 0, 3, 5, 10, 15 after initial ingestion. Kruskal-Wallis rank sum test followed by Game-Howell was conducted for (A), (B), (C), (D), and (E). *P<0.05, **P<0.01, ***P<0.001.

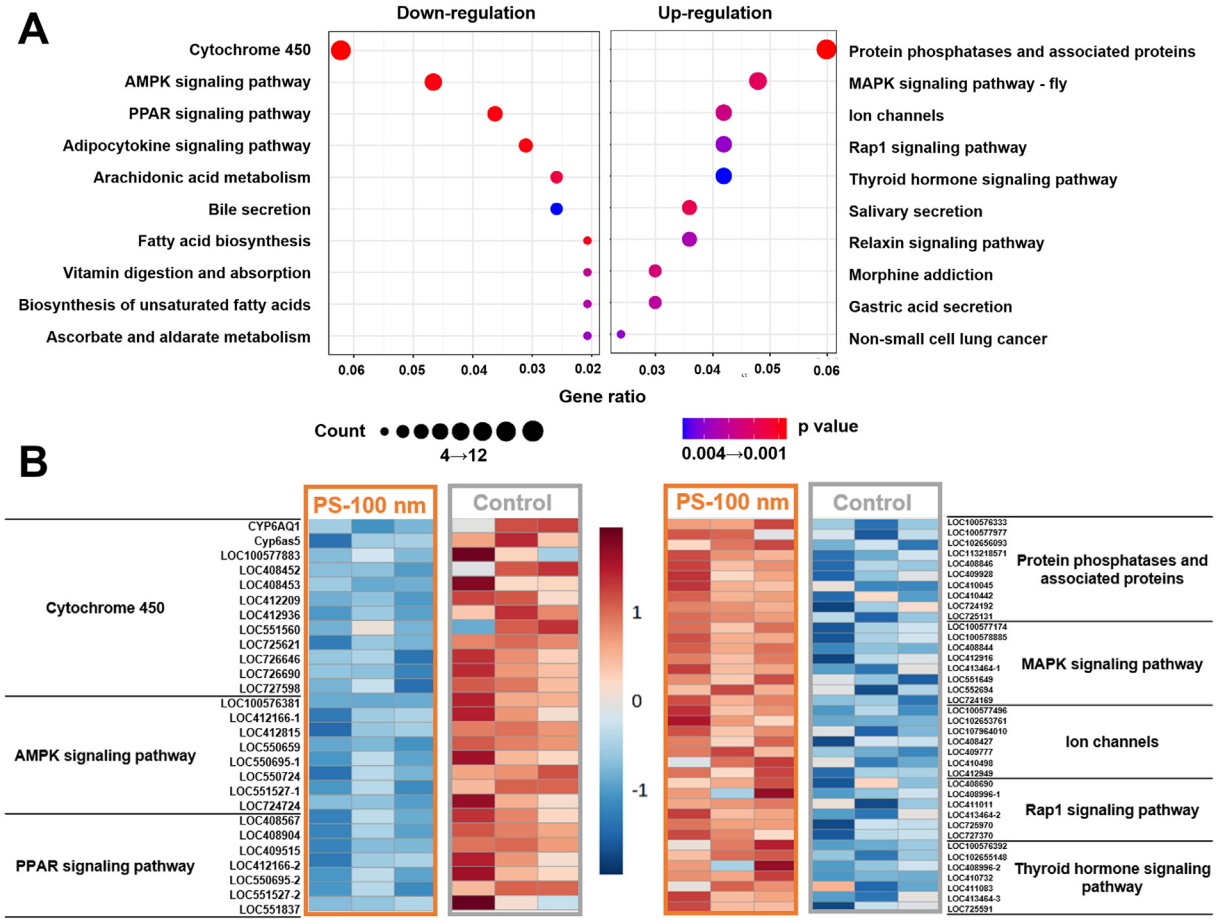

Fig. 6. Transcriptional profiles of the gut epithelial cells of honeybees on Day 15 after initial exposure. (A) The down-upregulated and up-regulated KEGG pathways on PS-100 nm compared to the control group. (B) Heatmap showing differentially expressed genes of honeybees from control and PS-100 nm groups. Each column represents one honeybee's sample.

than those from the "Control" and the "*H. alvei*" group, respectively. Accordingly, the abundance of *H. alvei* in guts was significantly increased to 11.83 % in the "PS-100 nm + *H. alvei*" group. At the same time, *H. alvei* was barely detected in the bee gut from the "Control," the "PS-100 nm", and the "*H. alvei*" groups (Fig. 7C), indicating that the 100-nm PS treatment significantly increased the abundance of *H. alvei* in guts.

Further, to understand whether the enhanced load of pathogenic bacteria in the guts of honeybees exposure to 100-nm PS would have adverse impacts on gut development, H&E staining of midguts of the honeybees was performed (Fig. 7D–G). As shown in Fig. 7D, the midgut epithelial cells of honeybees from the "Control" group appeared to develop well, with the multi-layered peritrophic membrane intact and food particles found scattered around the epithelial cells. These epithelial cells exhibited regular cell boundaries with normal nuclei. The columnar cells have the most quantity among epithelial cells found in the honeybees, and they form a single layer on the basement membrane. However, as shown in Fig. 7G, adverse effects were severe in honeybees in the "PS-100 nm + *H. alvei*" group, where the peritrophic membrane was utterly raptured. Incomplete basement membrane, nonvisible nuclei, cytoplasmic vacuolation, and lysis were also observed. As shown in Fig. 7E–F, adverse effects were alleviated, and the peritrophic membrane appeared to be intact and transparent but with reduced thickness to only one layer. Columnar cells had multiple small vacuoles, and the basement membrane was compromised. The measurement of intestinal wall thickness showed that honeybees from the "Control", "PS-100 nm", and "*H. alvei*" groups had thicker intestinal walls while bees in the "PS-100 nm + *H. alvei*" group had thinner intestinal walls (Fig. 7H). Unlike the significant difference in intestinal wall thickness between the PS-100 nm and control group in Fig. 2E (date obtained on Day 15), those between "Control" and "PS-100 nm" are not significant in Fig. 7H (obtained on Day 10), suggesting that effect of nanoplastics on the morphology of honeybee intestine was not fully realized yet on Day 10. The reduced thickness of the intestinal wall facilitated the exudation of gut contents, which may provide an opportunity for the pathogenic bacteria to invade from guts into other tissues. Accordingly, no bacterial colony was found in the hemolymph of honeybees in the "Control" (Fig. 7I), "PS-100 nm" (Fig. 7J), and "*H. alvei*" groups (Fig. 7K). In contrast, the live bacterial colonies were obtained in the hemolymph of honeybees from the "PS-100 nm + *H. alvei*" group, later identified as *H. alvei* by sequencing (Fig. 7L).

4. Discussion

Recently, there has been an increasing concern about the hazardous impact of N/MPs on the environment. Honeybees have been used as bioindicators for pollutants due to their apparent advantages, especially their abilities to illustrate the effect of gut microbiota on their hosts' conditions. In this study, we examined how plastic particles' sizes affect bees' growth and health. The aforementioned weight change results suggest that only the ingestion of 100-nm PS has decreased the weight change of honeybees. In contrast, ingestion of 1- and 10-μm PS presented a non-lethal effect. Another study showed that 25-μm PS had no changes to the weight gains in honeybees (Wang et al., 2020). It is in agreement with our results of MPs, but nanoscale microplastics affect the weight change of honeybees. The decrease in body weight was also observed in other

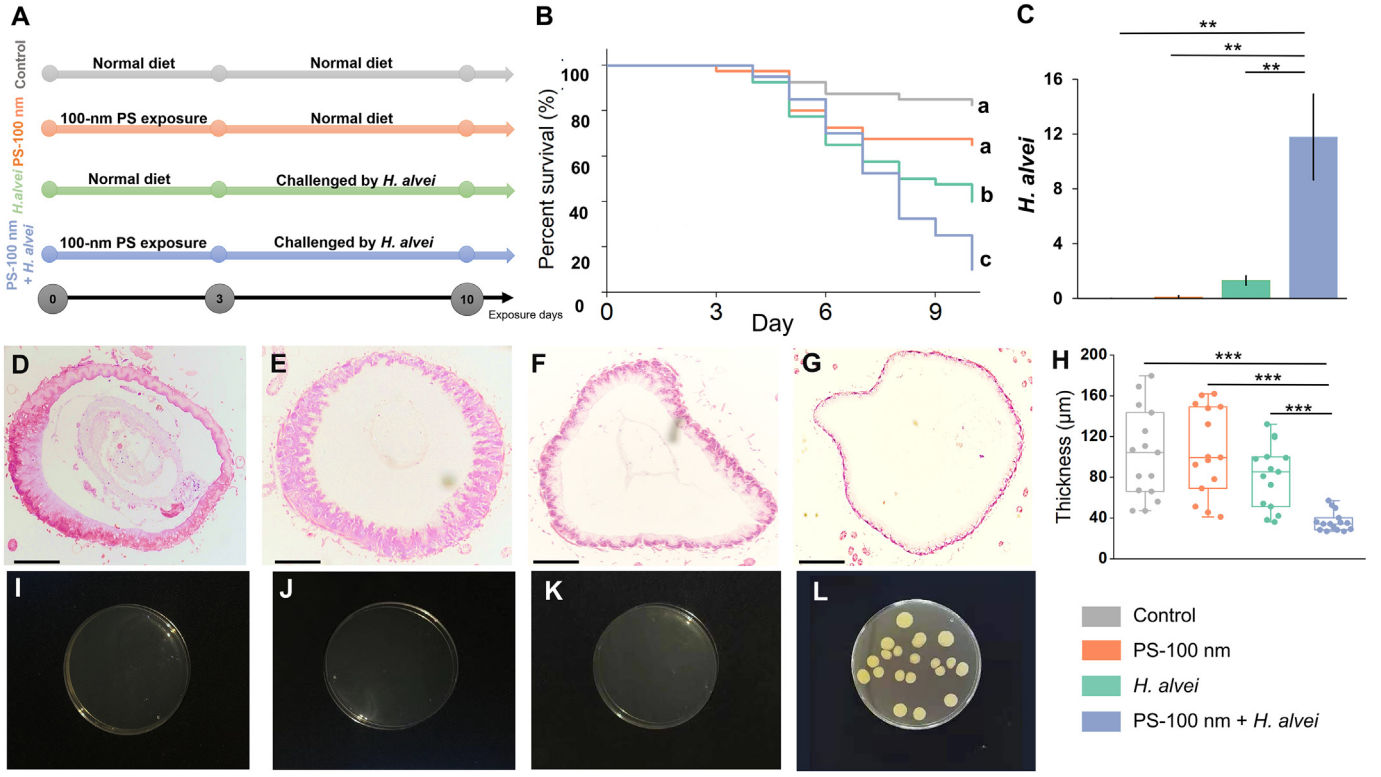

Fig. 7. Effects of *H. alvei* on the honeybee pre-exposure to 100 nm PS. (A) Experimental design of the susceptibility of honeybees to *H. alvei* after pre-exposure of NPs. (B) Kaplan-Meier survival curve of honey bees from "Control", "PS-100 nm", "*H. alvei*", and "PS-100 nm + *H. alvei*" groups. (C) The abundance of *H. alvei* in guts of honeybees. H&E staining of guts of honeybees from (D) "Control", (E) "PS-100 nm", (F) "*H. alvei*", and (G) "PS-100 nm + *H. alvei*" groups. (H) Boxplots of the intestinal wall thickness of honeybees from different groups. Plate culture of hemolymph of honey bees from (I) "Control", (J) "PS-100 nm", (K) "H. alvei", and (L) "PS-100 nm + *H. alvei*" groups. (C)-(L) were all obtained from honeybees in different groups on Day 10 after initial ingestion. Kaplan-Meier survival model with Log-rank test was used for (B). Eight replicates were performed for each group. Mauchly method was used first, and then MANOVA was conducted for (C) and (H). The length of the scale label is 100 μm. **P*<0.05, ***P*<0.01, ****P*<0.001.

species, such as the fruit fly, seabird, lugworm, and soil oligochaete, after MP treatment (Besseling et al., 2013; Horton et al., 2020; Sara et al., 2021; Spear et al., 1995; Zhu et al., 2018). For lugworms, food dilution led to a lower feeding efficiency, and reduced feeding activity directly caused severe weight loss. The relative organic matter of the sediment decreased 5.3 % owing to the addition of MPs, suggesting that an elevated volume of food source was required for the lugworms to gain the same amount of nutrition. It is evidenced that MPs pollution negatively impacted lugworms' growth by interference with their food availability (Besseling et al., 2013). However, sufficient food was provided to the honeybees in our study, with or without N/MPs exposure, and the food dilution was neglectable. Therefore, the presence of NPs, not MPs, may pose other adverse health impacts on honeybees, ultimately being reflected as weight loss.

After N/MPs enter into organisms, the accumulation of MPs has been reported in zebrafish and mice's guts and subsequently caused mucosal damage and other adverse health effects such as increased friction, inflammation, and metabolic disruption (Deng et al., 2020; Qiao et al., 2019). We observed the distribution of 10-μm PS in the guts of honeybees over time. MPs moved from the midgut to the end of the rectum and eventually accumulated in the rectum. Since the rectum harbors abundant gut microbiota (Martinson et al., 2012; Ricigliano and Anderson, 2020), the accumulation of MPs may affect the gut microbiota. Another study showed fluorescence signals of MPs were observable in the whole digestive tract in honeybees exposed to 0.5- and 5-μm PS for 21 days (Deng et al., 2021). The different particle sizes of MPs may have resulted in the different distribution of MPs in bees' guts.

The thickness of the intestinal wall of honeybees from the PS-100 nm group significantly decreased. Poorly developed intestines have thinner intestinal walls and deeper crypts, which are not conducive to digestion and absorption. Hence, these results demonstrate that ingestion of 100-nm PS hampered the normal development of the midgut of the honeybee and may eventually cause maldevelopment. The damage to the gut caused by NPs leads to poor absorption and digestion, resulting in weight loss.

Healthy and balanced gut microbiota is a crucial factor for the digestion and absorption of food, as well as maintaining the normal body weight gain of honeybees (Zheng et al., 2017). We found a significant interaction between N/MPs, pollen, and gut microbiota through SEM observation of gut homogenate. Pollen is a vital component of the honeybees' diet, which is their only food source of proteins, lipids, vitamins, and minerals (Frias et al., 2016). However, it has a highly recalcitrant external structure that prevents nutrients inside from being completely digested in the gastrointestinal tract (Zuluaga-Domínguez et al., 2019). At present, the research on the digestion process of pollen in honeybees is still unclear, with two theories recognized by the scientific community. One theory states that the pollen contents need to be released from the germination pore of pollens into the gut before being digested by enzymes (Dobson and Peng, 1997; McKinstry et al., 2020), while the other theory proposes that digestive enzymes in the gut can enter the pollen through the germination pore for digestion (Wright et al., 2017). In both theories, the key to pollen digestion lies in the direct contact of digestive enzymes with the contents inside pollen. When NPs exist in the germination pores of pollen grains, it may prevent the digestive enzymes from coming into contact with the pollen contents, making it difficult to digest the pollen fully. The MPs are larger, so no MPs were observed in the germination pore of pollen grains. In contrast, a substantial amount of microorganisms in the honeybee's gut were found on the MPs due to their large surface area, which was consistent with a previous study (Wang et al., 2020). Environmental microorganisms

were reported to reside on the surface of MP found in intertidal ecosystems (Jiang et al., 2018), urban rivers (McCormick et al., 2014), oceans (Arias-Andres et al., 2019), and soil (Chai et al., 2020). MP's hard and hydrophobic surface and the nutrients absorbed from the environment made them preferred habitats for specific microorganisms. Interestingly, besides bees, colonization of gut microorganisms on microplastics was also observed on MPs in shrimps (Yan et al., 2021), which altered the composition of gut microbiota of the host and incited subsequent adverse health effects.

Recently, the contributions of individual microbiota members to nutrition digestion have been studied (Zheng et al., 2019). Among bee gut microbes, the *Bifidobacterium* possesses the most abundant and diverse genes for carbohydrate utilization. Bee-associated *Lactobacillus* have numerous phosphotransferase systems that are essential in sugar uptake (Ellegaard et al., 2015). Since honeybees have a diet rich in carbohydrates, these two microorganisms who benefit most from this food are often dominant in the gut microbiota (Kwong and Moran, 2016). In the study, *Bifidobacterium* and *Lactobacillus* decreased after 100-nm PS exposure. We speculated that germination pores of pollen grains were adhered to and pre-occupied by the 100-nm PS, so the pollen utilization by gut microbiota was hindered. Restricted access to the necessary nutrients from pollen could cause the decrease of *Lactobacillus* and *Bifidobacterium* (Fig. 5). The imbalance of gut microbiota will lead to a decline in host function, such as immune function (Zheng et al., 2018), which may be one of the reasons for gut maldevelopment. However, 1- and 10-μm PS mainly occupied the living space of gut microflora without altering the structure of gut microbiota, and the related effects were not apparent. Hence, we interpreted that the pollen adhesion by NP may contribute more to the effect by interfering with the nutrient intake of the bacteria.

Since all of our results showed that NPs posed a more obvious threat to honeybees than MPs, transcriptome analysis was conducted on bees in the NPs group. Up-regulated pathways in the PS-100 nm group mainly include protein phosphatases and associated proteins, as well as the mitogen-activated protein kinase (MAPK) signaling pathway. MAPK signaling pathway is evolutionarily well conserved in all eukaryotic cells, including a MAP kinase kinase kinase (MAP3K) (Matsuzawa and Ichijo, 2008). Apoptosis signal-regulating kinase 1 (ASK1) belongs to MAP3K, required for innate immune response, which is preferentially activated in response to various types of stress such as oxidative stress (Matsuzawa and Ichijo, 2008). The dual-specificity protein phosphatase family exerts its effects by blocking ASK1 phosphorylation (Ye et al., 2019), and the serine/threonine phosphatase family binds to and dephosphorylates the activated form of ASK1 as well (Morita et al., 2001). Our results showed that the LOC408844 gene in MAPK signaling pathways, which encodes the dual-specificity protein phosphatase, significantly increased after the PS-100 nm treatment. Correspondingly, LOC410045 related to serine/threonine-protein phosphatase four protein in protein phosphatases and associated proteins pathways also increased after PS-100 nm treatment. These suggest that 100-nm PS decreased the immune response of bees, which is not conducive to the resistance to various stress. Hence, when challenged by external stress, such as pathogens, the honeybee with an undermined immune system would be more susceptible to infections (Fig. 7).

Down-regulated pathways in the PS-100 nm group include cytochrome P450 and AMPK signaling pathways. Cytochrome P450 is a kind of microsomal multifunctional end-oxidase containing heme and thiol, mainly involved in synthesizing and decomposing exogenous and endogenous compounds in organisms (Nelson and Strobel, 1987). Previous studies have highlighted their essential roles in detoxification (Johnson et al., 2006). We found that the expression of CYP6AQ1 in the cytochrome P450 pathway decreased with the 100-nm PS treatment, probably leading to reduced detoxification capabilities in honeybees. AMP-activated protein kinase (AMPK, a serine/threonine kinase) is responsible for regulating food intake by integrating nutrient and hormonal signals (Ruderman et al., 2013), and it can sustain the energy balance by suppressing processes that consume a high level of ATP (Ke et al., 2018). In our results, genes in AMPK signaling pathways significantly were down-regulated after PS-100 nm treatment, interfering with the energy balance. Hence, the AMPK signaling pathways would not be activated, leading to the failure of ATP preservation during energy deficiency in honeybees. The unmediated energy consumption led to energy loss and eventual weight loss, as we observed in Fig. 1B.

In this study, we also investigated the effect of pathogens on the NP/MP compromised honeybees as the spread of parasites and pathogens is one of the key factors causing high colony mortality (Dolezal and Toth, 2018; Evans and Schwarz, 2011). Among the *Enterobacteriaceae* species that occur sporadically in bee gut, *H. alvei* is considered an opportunistic pathogen as it was isolated from the intestines and tissues of dead bees in fatal cases of septicemia (Padilla et al., 2014; Tian and Moran, 2016). Recently, Lang et al. reported that the *Lactobacillus apis* W8172 could protect honeybees from the deadly infection of *H. alvei* through stimulation of the host immune system (Lang et al., 2022). It supported our findings that NPs-exposed bees were significantly more susceptible to *H. alvei,* possibly due to the disturbance of the gut microbiota. The decreased abundance of *Lactobacillus* presented in Fig. 5 suggested that the ingestion of NPs compromised its pathogen invasion protection function. A good balance is generally maintained between the normal gut microbiota and the host through nutritional competition and mutual restriction of metabolites and other factors. But this balance could be broken when facing stress, leading to the growth of opportunistic pathogens and the associated adverse impacts on living organisms (Klainer and Beisel, 1969).

In addition, after exposure to NPs, the pathogenic bacteria resulted in significant thinning of the intestinal wall of the bees, which may allow the pathogenic bacteria to infect its hemolymph (Gwak and Chang, 2021). Hemolymph is a major extracellular fluid in the circulatory system of honeybees, whose functions are equivalent to the blood and lymph of vertebrates (Svečnjak et al., 2012). It is closely related to the immune response of honeybees to various negative factors, thus reflecting the body's physiological state (Horvatinec and Svečnjak, 2020). As aforementioned, the presence of NPs could interfere with the genes related to the immune response. Consequently, the undermined immune system failed to prevent the uncontrolled reproduction and widespread of pathogenic bacteria. Overall, the NPs make honeybees more susceptible to pathogenic bacteria, resulting in increased mortality in honeybees from the “PS-100 nm + *H. alvei* group”. N/MPs in the environments can serve as hotspots for the enrichment and spread of multiple pathogens, as well as a vector for metals, antibiotics, and toxic chemicals (Ravidas et al., 2019; Wang et al., 2021). This feature would be potentially lethal to living organisms exposed to a complex environment compared to NPs alone, as its existence might increase the adverse impacts of other pressures on organisms. It is evident in our study that the gut microbiota and food source are key components for unveiling the mechanism of the health implication of MP/NP on honeybees. Future studies on the effect of nano- and microplastics on specific honeybee gut microorganisms and the metabolites are needed.

5. Conclusion

In this study, we found that the ingestion of nano-polystyrene plastic caused adverse health conditions in honeybees. After PS with a diameter of 100 nm and a concentration of 10^5 particles/ml was ingested by honeybees, they accumulated in the germination pore of pollen in the rectum. It might hinder the enzyme from contacting pollen and interfere with utilizing nutrients in pollen by gut microbiota. An imbalanced gut microbiota would cause gut maldevelopment and inflammation, leading to decreased digestion and absorption capacity of honeybees. Ultimately, the energy deficiency and significant weight loss in honeybees appeared. In addition, PS with a diameter of 100 nm could increase the reproduction of *H. alvei* in guts and promote the spread of pathogenic bacteria from guts to hemolymph, causing the increased mortality of honeybees. Therefore, exposure to nano-polystyrene plastic compromised the normal growth of honeybees, and its existence increased the adverse impacts of other stress on organisms. The present study extended and complemented the current understanding regarding the health impact of the emerging environmental pollution of plastic debris, especially on the nanoscale.

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CRediT authorship contribution statement

Kewen Wang: Sampling and investigation, Formal analysis, Data curation, Writing - review & editing. **Liya Zhu:** Sampling and investigation, Formal analysis, Data curation, Writing - review & editing. **Lei Rao:** Conceptualization, Funding acquisition. **Liang Zhao:** Conceptualization, Funding acquisition. **Yongtao Wang:** Conceptualization, Funding acquisition. **Xiaomeng Wu:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Hao Zheng:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Xiaojun Liao:** Conceptualization, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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