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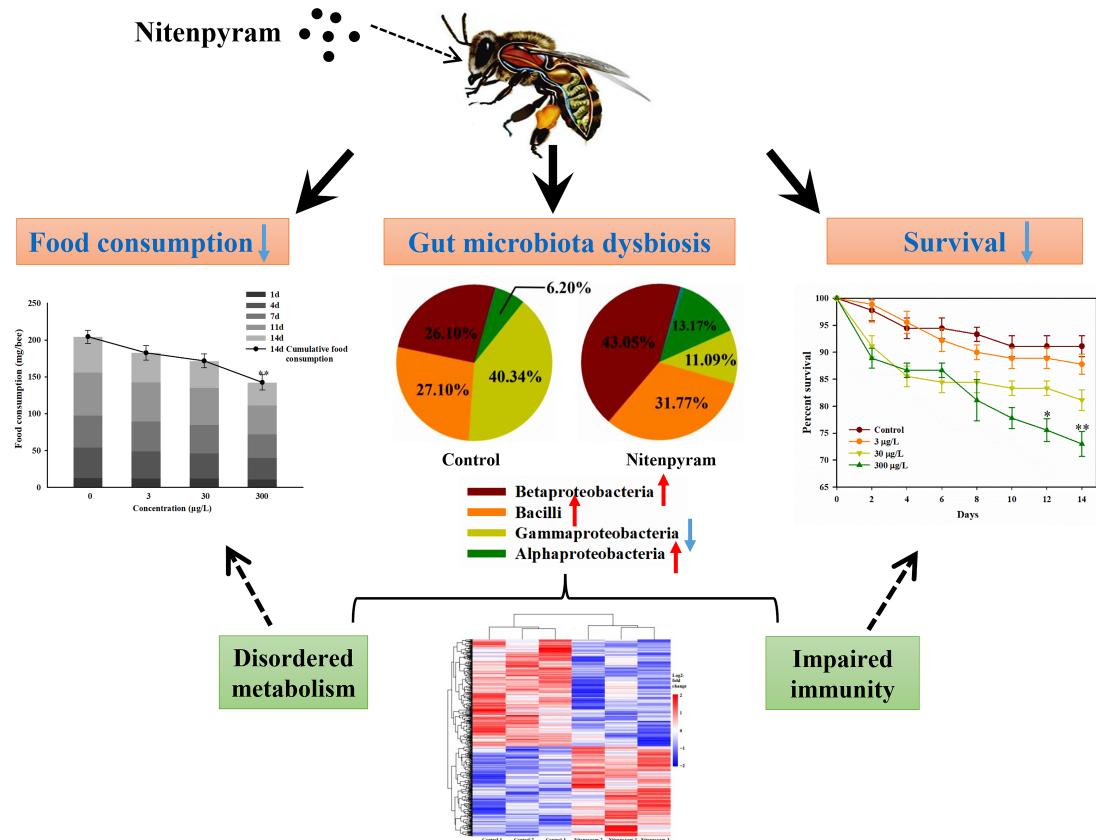
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1 **Nitenpyram disturbs gut microbiota and influences metabolic homeostasis**

2 **and immunity in honey bee (*Apis mellifera L.*)**

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26 **Abstract**

27 Recently, environmental risk and toxicity of neonicotinoid insecticides to honey
28 bees have attracted extensive attention. However, toxicological understanding of
29 neonicotinoid insecticides on gut microbiota is limited. In the present study, honey
30 bees (*Apis mellifera L.*) were exposed to a series of nitenpyram for 14 days. Results
31 indicated that nitenpyram exposure decreased the survival and food consumption of
32 honey bees. Furthermore, 16S rRNA gene sequencing revealed that nitenpyram
33 caused significant alterations in the relative abundance of several key gut microbiotas,
34 which contribute to metabolic homeostasis and immunity. Using high-throughput
35 RNA-Seq transcriptomic analysis, we identified a total of 526 differentially expressed
36 genes (DEGs) that were significantly altered between nitenpyram-treated and control
37 honey bee gut, including several genes related to metabolic, detoxification and
38 immunity. In addition, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and
39 Genomes (KEGG) pathway analysis showed nitenpyram affected several biological
40 processes, of which most were related to metabolism. Collectively, our study
41 demonstrates that the dysbiosis of gut microbiota in honey bee caused by nitenpyram
42 may influence metabolic homeostasis and immunity of bees, and further decrease
43 food consumption and survival of bees.

44 **Capsule:** Nitenpyram affected metabolism and immunity putatively caused by
45 disordered gut microbiota, further decreased food consumption and survival of bees.

46 **Keywords:** Nitenpyram; Honey bee; Gut microbiota; Metabolism; Immunity

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53 **1. Introduction**

54 The honey bee, *Apis mellifera*, is the most important pollinator for agricultural
55 and natural ecosystems in the world and contributes significantly to the human food
56 supply (Hung et al., 2018). As forage insects, when they gather nectar and pollen,
57 bees are particularly vulnerable to sublethal toxin exposures (Zhang et al., 2017).
58 Among the agrochemicals known to be toxic to bees, neonicotinoid insecticides are a
59 class of widespread and fastest-growing insecticides worldwide (Decourtye and
60 Devillers, 2010). It has been demonstrated that neonicotinoid insecticides have a
61 series of adverse effects on honey bees and other pollinators, including negatively
62 affecting behavior (Axel et al., 2003), learning (Guez et al., 2001), memory (Hassani
63 et al., 2008), immunocompetence (Brandt et al., 2016), orientation and foraging (Yang
64 et al., 2008) of worker bees, reducing the reproductive anatomy and physiology of
65 honey bee queens (Williams et al., 2015).

66 Nitenpyram[(E)-N-(6-Chloro-3-pyridylmethyl)-N'-ethy-N'-methyl-2-nitrovinylid
67 enediamine], a member of the most widely used second-generation neonicotinoid
68 insecticides, was developed and commercialized by Takeda Chemical Industries, Ltd.
69 in 1989 (Alfred et al., 2010). Like other neonicotinoids, nitenpyram acts as agonist of
70 the nicotinic acetylcholine receptor (nAChR) of insects, which plays an important role
71 in the mediation of fast excitatory synaptic transmission in the insect central nervous
72 system (CNS) (Hirotaka et al., 2003). In recent years, nitenpyram has been commonly
73 used to control pests for rice, maize, sunflowers, rape, vegetables, and fruits crops,
74 and achieved satisfactory effect in agricultural production (Dong et al., 2014). In
75 addition, nitenpyram has also been used to control fleas in dogs and cats (Correia et

76 al., 2010). Nevertheless, with its widespread use and highly water-soluble nature
77 (Morrissey et al., 2015), nitenpyram can transfer into environments easily. A previous
78 study had proved that nitenpyram was found in pollen detected from hives located
79 within 30 km of the City of Saskatoon, Saskatchewan, Canada (Codling et al., 2016).
80 Similarly, a recent study reported that nitenpyram residue was detected in 30 honey
81 samples collected from different regions of China and its residue level was 13-41
82 µg/kg (Song et al., 2018). Moreover, it was reported that nitenpyram had adverse
83 effects to non-target organisms. Wu et al. determined acute toxicity of nitenpyram to 6
84 kinds of non-target organisms and found that nitenpyram was middle toxic to
85 earthworm (*Eisenia foetida*), but highly toxic to honey bees (*Apis mellifera L.*) and
86 silkworm (*Bombyx mori*) (Wu et al., 2016). Therefore, more attention should be paid
87 on the potential hazards of nitenpyram to the environmental organisms and its toxic
88 effects.

89 The gut microbiota plays an essential role on the maintenance of host health. In
90 honey bees, the gut microbiota is relatively simple and conservative, which confers
91 functions including food processing, regulation of immune system, and defense
92 against pathogens (Philipp et al., 2013). Therefore, studying the effects of pesticides
93 on gut microbiota will be helpful to explore the mechanism of action of pesticides and
94 develop new sensitive biomarkers and methods, as well as develop exogenous
95 compensatory nutrition to reduce the effects of drugs, which will be benefit to the
96 usage of pesticides and the protection of bees. Previous studies had shown that several
97 pesticides could affect midgut bacterial communities of honey bee during adult
98 workers (Motta et al., 2018) and larvae (Dai et al., 2018a). However, limited studies
99 have been conducted on the negative effects of other neonicotinoid insecticides
100 exposure on honey bee gut microbiota, only a recent study found that imidacloprid

101 decreased honey bee survival rates but didn't affect the gut microbiome (Raymann et
102 al., 2018). Therefore, it is necessary to perform environmental toxicological studies of
103 neonicotinoid insecticides to reflect its environmental risk on honey bee gut
104 microbiota. In the present study, honey bees were exposed to a series of
105 concentrations of nitenpyram for 14 days to evaluate the survival, food consumption
106 and gut bacterial communities, as well as exploring the impact on gut gene profiles in
107 honey bees. Overall, our results show that nitenpyram exposure can perturb the gut
108 microbiota of honey bees, further affect metabolic homeostasis and immunity. It is
109 believed that the results could provide some new information on neonicotinoid
110 insecticides-induced toxicity in honey bees.

111 **2. Materials and methods**

112 **2.1. Chemicals and reagents**

113 Nitenpyram (CAS: 150824-47-8; 95% purity) was obtained from the Chinese
114 Ministry of Agriculture. With high water solubility (590.000mg/L @ 20°C) and stable
115 water hydrolysis under acidic and neutral pH conditions (Morrissey et al., 2015),
116 nitenpyram was dissolved in 50% sucrose solution. The treatment solutions were
117 freshly prepared each day. Other chemicals were all analytically pure and purchased
118 from Beijing Chemical Co. (Beijing, China).

119 **2.2. Maintenance of honey bees and toxicity test**

120 Experiments were carried out with worker bees of *Apis mellifera L.*, which were
121 maintained according to standard beekeeping practices (Wu et al., 2017), in an apiary
122 at the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences,
123 Beijing. Prior to our study, there was no hive treatments performed to control disease.
124 Before the experiment, newly emerged bees were collected for routine testing with
125 standard RT-PCR assays for identification of common bee viruses according to a

126 previous study (Fredrik et al.). The experiment began on May 20 and finished on
127 September 10, 2018.

128 For acute toxicity test, newly emerged bees were collected from the brood combs
129 from three colonies and randomly divided in plastic containers with mesh on two
130 sides. Although it has been well known that newly emerged bees consume pollen to
131 enable further development, it was proved that pollen could modulate cytochrome
132 P450 (CYP) detoxication enzymes (Carla et al., 2016). For avoiding interfering the
133 effect of nitenpyram on the bees, the bees were only fed 50% sucrose solution
134 throughout the experiment. A syringe containing 2mL the nitenpyram treatment
135 solution was inserted above the plastic container to allow the bees to feed freely. The
136 treatment consisted of six concentrations of test solutions (1.0, 2.0, 3.0, 4.0, 5.0 and
137 6.0 mg/L) designed on the basis of pre-experimental data, and 50% sucrose solution
138 served as the blank control (0 μ g/L). The treatment solutions were all prepared with
139 50% sucrose solution and renewed every 24 h. Three replicates were run for each
140 treatment, including 10 worker bees in each replicate. The bees were kept in an
141 incubator ($30\pm2^\circ$, 50±10% relative humidity, darkness) for 48h. The mortality of
142 honey bees were calculated daily.

143 For chronic toxicity studies, newly emerged bees from three colonies were
144 randomly assigned to plastic containers with syringes inserted above them, as same as
145 the bees used in the acute toxicity test in the current study. Each syringe contained
146 2mL nitenpyram treatment solution. Bees were continuously fed 50% (w/v) sucrose
147 solution containing concentrations of 3, 30, 300 μ g/L of nitenpyram for 14 days, which
148 were almost be the 1/1000, 1/100, 1/10 of the LC₅₀ obtained from the acute toxicity
149 test. 50% sucrose solution served as the over-all control (0 μ g/L). Three replicates
150 were run for each treatment, including 30 worker bees in each replicate. The treatment

151 solutions were updated every 24 h. The dead bees in each group were recorded and
152 the survival rate was calculated. The food consumption test was conducted in
153 accordance with a previously proposed method (Yang et al., 2019). The amounts of
154 solutions in the syringes were weighed daily before they were placed in the containers
155 and again after they were removed from the containers. The difference was equivalent
156 to the total amount of food consumed by live bees on the previous day, and then
157 calculating the amount of food consumed by each honey bee. After pesticide
158 treatment, midgut of worker bees were collected in 1.5-ml centrifuge tubes and
159 immediately frozen in liquid nitrogen and preserved at -80°C until further analysis.

160 2.3. Gut microbiota analysis

161 From each treat group, 6 midgut were pooled together as a biological sample and
162 three replicates were used (n=3 per treatment group). Genomic DNA was extracted by
163 Toptaq DNA polymerase kit (Transgen, China), according to the manufacturer's
164 instructions. All the extracted gDNA was quantified by ultraviolet spectroscopy for
165 further analysis. Next, the microbial gDNA was amplified by specific primers
166 (Forward primer: 5'-CCTACGGNGCWGCAG-3' ; Reverse primer : 5'-
167 GACTACHVGGTACTATCC-3') targeting the V3 and V4 regions of the bacterial 16S
168 rRNA gene. Furthermore, the sequencing and bioinformatics were implemented by
169 Genesky Biotechnologies Inc. (Shanghai, China) on the Illumina MiSeqBenchtop
170 Sequencer (Illumina, USA) to generate 2 ×250 bp paired-end reads. The effective tags
171 were then clustered by UPARSE to Operational Taxonomic Units (OTUs) according
172 to 97% similarity. After classification annotation based on Ribosomal Database
173 Project (RDP), the numbers of sequences for each classification level (e.g. phylum
174 and genus) were calculated and summarized to compare the abundance and diversity
175 of gut microbiota between exposed groups.

176 2.4. RNA-Seq analysis of differentially expressed genes

177 From each treat group, 6 midgut were pooled together as a biological sample in
178 each treatment group and three replicates were used (n=3 per treatment group).
179 TRIZol reagents (Tiangen Biotech, Beijing, China) were used to extract total RNA
180 from the frozen midgut according to the manufacturer's protocol. RNA quality and
181 concentration were checked using the NanoPhotometer spectrophotometer (IMPLEN,
182 CA, USA). In accordance with manufacturer's recommendations, NEBNext UltraTM
183 RNA Library Prep Kit for Illumina (NEB, USA) were used to generate sequencing
184 libraries, adding indexes in every sample code to attribute sequences to each sample.
185 The clustering of the index-coded samples was implemented on a cBot Cluster
186 Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) following the
187 manufacturer's instructions. FeatureCounts v1.5.0-p3 was used to count the reads
188 numbers mapped to each gene. Normalizing of the differential expression analysis
189 was performed by using DESeq2 R package (1.16.1) among treatments. Genes with
190 an adjusted P-value <0.05 found by DESeq2 were assigned as differentially
191 expressed. Gene Ontology (GO) enrichment analysis of differentially expressed genes
192 was carried out by the clusterProfiler R package, which was also used to test the
193 statistical enrichment of differential expression genes in KEGG pathways.

194 2.5. Gene expression analysis

195 Fifteen DEGs (BMP10, HRG, SMYD4, H2A, maltase A3, OR4, pro-resilin,
196 FLO11, TLR4, Secapin, FAXDC2, CCKR, CYP6a14, UTP18 and E4) were selected
197 for real-time quantitative PCR (qPCR) assays. The details on cDNA synthesis and the
198 real-time PCR were performed as described previously (Zhu et al., 2015). The
199 transcription of β -actin was used as a house-keeping gene. The primers used in this
200 study are listed in Table S1. The fold-change of the genes tested was calculated using

201 the $2^{-\Delta\Delta Ct}$ method.

202 **2.6. Statistical analysis**

203 SPSS16.0 (SPSS, Chicago, IL, USA) were used for analyzing the mortality, food
 204 consumption and survival experiments. Differences were determined by one-way
 205 ANOVA, completed with Dunnett and Duncan post-hoc comparison. $P<0.05$ was
 206 considered statistically significant. Mothur was used to calculate rarefaction analysis,
 207 the community richness index, and alpha diversities including Shannon and Simpson
 208 indexes. Principal coordinate analysis (PCoA) and hierarchical cluster analysis were
 209 based on euclidean distance matrix and ward. D2 method was performed according to
 210 the abundances of operational taxonomic units (OTUs) by R package (V3.4.0).

211 **3. Results**

212 **3.1. Acute and chronic toxicity of nitenpyram to honey bees**

213 In the acute toxicity test, the mortality of honey bees at two time points (24 and
 214 48 hpf) is shown in Fig. 1A. The results showed that nitenpyram exposure caused
 215 mortality of honey bees in a concentration-dependent manner. When the concentration
 216 of nitenpyram was 3.00mg/L and below, there was no significant difference in
 217 mortality rate between the control group and the nitenpyram group at 24h. While
 218 remarkable difference of mortality was observed at concentrations of 3.00mg/L and
 219 higher, with almost all bees exposed to nitenpyram at 6.00 mg/L dying at 48 hpf (Fig.
 220 1A). The LC₅₀ value of nitenpyram at 48 hpf was 3.37mg/L (Table S2).

221 In the chronic toxicity test, the survival rate of bees in control group was above
 222 90%, and the survival rates of bees in concentrations of 3 μ g/L and 30 μ g/L group were
 223 not significantly different from that of control group (Fig. 1B). While the survival
 224 rates of the bees in 300 μ g/L nitenpyram-treated group at 12 and 14 d were lower than
 225 that in control group, with survival rates as low as 76.6% and 73.3%, respectively (Fig.

226 1B).

227 After exposure to nitenpyram for 14d, the cumulative food consumption of
228 honey bees was also interfered observably. As shown in Fig. 1C, compared with
229 control group, there was no significant difference in the cumulative food consumption
230 at concentrations of 3 and 30 μ g/L nitenpyram. (Fig. 1C). While the cumulative food
231 consumption of honey bees exposed to 300 μ g/L nitenpyram was significantly lower
232 than that of bees exposed to control group (Fig. 1C).

233 3.2. Effects of nitenpyram on the honey bee gut bacterial community

234 To investigate the effect of nitenpyram on the changes of the gut microbiota, the
235 midgut microbiota of bees exposed to control and 300 μ g/L nitenpyram for 14 days
236 were analyzed (n=3). Through performing the 16S rRNA pyrosequencing based on
237 V3-V4 region, a total of 569,113 high-quality sequences were acquired from 6
238 samples, with an average length of effective sequences reads of 420 bp. A maximum
239 of 110 unique operational taxonomic units (OTUs) were clustered based on 97%
240 similarity cut-off. Among them, there were 68 OTUs detected both in control and
241 300 μ g/L nitenpyram-treat group, while 8 OTUs were found only in control group and
242 34 OTUs in 300 μ g/L nitenpyram treat group.

243 As shown in Fig. 2A, at the phylum level, Proteobacteria, Firmicutes and
244 Actinobacteria were found to be the most abundant phyla in both control and 300 μ g/L
245 nitenpyram-treat group, and there was no significant difference in microbiota
246 composition between the two groups. (Fig. 2A). At class level, there was a marked
247 decrease of the abundance of Gammaproteobacteria, which was reduced from 40.34%
248 in the control group to 11.09% in the 300 μ g/L nitenpyram-treat group (Fig. 2B).
249 While the composition of Betaproteobacteria, Bacilli and Alphaproteobacteria
250 increased after nitenpyram exposure, and the former increased from 26.10% to

251 43.05%, the middle increased from 27.10% to 31.77%, the latter increased from
 252 6.20% to 13.17%, respectively (Fig. 2B). Moreover, at genus level, the abundance of
 253 *Frischella*, *Bartonella* and *Bifidobacterium* were all significantly increased, while that
 254 of *Gilliamella* was strikingly reduced after exposure to nitenpyram, which was almost
 255 completely absent in the 300 μ g/L nitenpyram-treatment group (Fig. 2C).

256 Four alpha diversity parameters including Chao1 index, ACE index, Shannon
 257 index and Simpson index, were employed for community richness comparisons
 258 (Table S3). And there were no significant difference in all of the four alpha diversity
 259 indices in communal richness nor diversity (Table S3). According principal
 260 coordinates analysis (PCoA) of weighted UniFrac, the gut microbiota of 300 μ g/L
 261 nitenpyram-treated group showed a deviation from the control group (Fig. 2D),
 262 however, the P value of PCoA was 0.20 obtained by ADONIS analysis, indicating the
 263 difference of the gut microbiota between two groups was not significant. The most
 264 abundant genera (top30) were displayed in Hierarchical cluster analysis, which also
 265 showed that the gut microbiota of nitenpyram-treated group differed from the control
 266 group (Fig. 2E). In addition, a linear discriminant analysis effect size (LEFSe)
 267 analysis was performed, which was shown in Fig. 2F. LEFSe analysis identified that
 268 *Gammaproteobacteria* (class), *Orbales* (order), *Orbaceae* (family), *Gilliamella* (genera)
 269 and *Gilliamella apicola* (species) were rich in control bees(Fig. 2F). While *Frischella*
 270 (genera) and *Frischella perrara* and *Lactobacillus helsingborgensis* (species) were
 271 rich in the 300 μ g/L nitenpyram-treatment group (Fig. 2F).

272 3.3. Raw Read Processing and Quantitative Gene Expression

273 To determine the impact of nitenpyram on gene expression of the honey bee gut,
 274 high-throughput RNA-seq was performed to analyze the global expression of
 275 differential expressed genes (DEGs) in gut of honey bees treated with nitenpyram

276 (300 μ g/L) for 14d compared with that in the control group (n=3). Each library
277 received more than 4.2 million raw reads, of which over 96.76% of these reads were
278 identified as clean reads (Table S4). After mapping to the reference genome (NCBI:
279 Amel_4.5), more than 92.83% successfully matched to either unique or multiple
280 locations of the honey bee genome (Table S4).

281 As shown in Fig.3A, the average numbers of genes expressed in nitenpyram and
282 control libraries were 9,481 and 9,402 respectively; and there were 9,265 genes
283 expressions expressed in both groups (Fig.3A). A total of 526 DEGs were detected in
284 honey bees exposed to 300 μ g/L nitenpyram, of which, 240 (45.6%) were upregulated
285 and 286 (54.4%) were downregulated (Fig.3B). All significantly differentially
286 expressed genes (SDEGs) were plotted in a heatmap for a crosswise comparison
287 among replicates relative to the average RPKM (Reads Per Kilobase per Million
288 mapped reads) between two DEG groups according to the similarity of their
289 expression patterns (Fig. 3C). Hierarchical clustering placed all three replicates of
290 control group bees within a single cluster, whereas 300 μ g/L nitenpyram-treated bees
291 formed another cluster. Using other clustering methods such as principal component
292 analysis (PCA) also obtained analogous results (Fig. 3D).

293 In addition to the uncharacterized genes, we also analyzed 20 known genes
294 which were most different in up-regulated and down-regulated expressions
295 respectively (Tables S5 and S6). These differential genes encoded proteins or enzymes
296 regulating diverse biological processes. For example, several genes involved in
297 metabolism were changed in honey bee gut exposed to 300 μ g/L nitenpyram, such as
298 PEPCK (Gene ID 412843), FAXDC2 (Gene ID 727357), and apolipophorins (Gene
299 ID 408961), etc (Table S5 and S6). Additionally, several genes related immunity and
300 pathogen defence were also significantly altered, such as Secapin (Gene ID 406145),

301 TLR4 (Gene ID 724187), endochitinase (Gene ID 551600), etc (Table S5 and S6).
302 Moreover, the expressions of several detoxification related genes were also found to
303 be affected in honey bee gut exposed to 300µg/L nitenpyram, for instance, CYP6a14
304 (Gene ID 724946), E4 (Gene ID 409801) and peroxidase-like (Gene ID 100577249),
305 etc (Table S5 and S6).

306 3.4. GO Functional Categories and KEGG Pathways

307 Based on the GO terms for biological process, 462 DEGs were divided into three
308 classes: molecular function, cellular components, and biological process. The top 30
309 most enriched terms are shown in Fig.S1, in which most of genes were enriched
310 coded for cellular components, including protein heterodimerization activity,
311 transporter activity, protein dimerization activity, heme binding and tetrapyrrole
312 binding (Fig. S1).

313 The KEGG database was used for annotation of DEGs distribution function.
314 There were 84 DEGs identified and mapped to 68 pathways in the KEGG pathway
315 database. Nine most significant enriched-pathways were listed in Table 1, including
316 the regulation of most genes related to pentose and glucuronate interconversions, drug
317 metabolism, ascorbate and aldarate metabolism, glycerophospholipid metabolism,
318 starch and sucrose metabolism, sphingolipid metabolism, tryptophan metabolism and
319 porphyrin, chlorophyll metabolism and retinol metabolism (Table 1).

320 3.5. Real-Time qPCR Analysis

321 To verify the results of the RNA-Seq data, we selected fifteen DEGs, of which 7
322 were from the most up-regulated genes and 8 were from the most down-regulated
323 genes for qPCR validation. Among them, most genes are involved in functions, such
324 as immunity, metabolism, detoxification and antioxidant reactions, which are
325 activated in response to environmental stressors in honey bees. As shown in Fig.4A

326 and 4B, all of the expression levels were significantly different in nitenpyram-treated
327 bees compared with controls (Fig. 4A and 4B). The expressions of BMP10, HRG,
328 SMYD4, H2A, maltase A3, OR4 and pro-resilin were downregulated in honey bees
329 exposed to 300 μ g/L nitenpyram (Fig. 4A), while that of FLO11, TLR4, Secapin,
330 FAXDC2, CCKR, CYP6a14, UTP18 and E4 were down regulated in honey bee gut
331 exposed to 300 μ g/L nitenpyram (Fig. 4B). The results were consistent with the
332 sequencing results.

333 **4. Discussion**

334 Currently, researches on the negative effects of neonicotinoid insecticides on the
335 physiological function of honey bees have been carried out extensively. Nevertheless,
336 understanding of the impact of neonicotinoid insecticides on gut microbiota,
337 especially its associated physiological activities, is lacking. To our knowledge, this is
338 the first study to analyze the changes in gut microbiota in honey bees exposed to
339 nitenpyram, as well as other negative effects correlated with these microbial changes.
340 From the data presented, it has been clearly indicated that nitenpyram exposure
341 decreased the survival and food consumption and greatly induced gut microbiota
342 dysbiosis in honey bees. Additionally, the transcriptome information of honey bee gut
343 under nitenpyram exposure is also presented for the first time, which provides the
344 molecular basis for the toxicity of nitenpyram to honey bee.

345 Acute doses of pesticides can kill individual honey bees and colonies outright,
346 while chronic exposure to low doses leads to sub-lethal effects in individual bees,
347 which, in turn, may result in colony-level effects. A recent study reported that
348 nitenpyram residue was detected in 30 honey samples collected from different regions
349 of China and the residue levels were range from 13 to 41 μ g/kg (Song et al., 2018). In
350 addition, the recommended quantity of nitenpyram in the field is range from 30 to 90

351 g ai/ha. Unfortunately, the exposure is usually not limited to the recommended dose,
352 with relatively higher concentrations applied in environment. In the present study, the
353 48 h-LC₅₀ value of nitenpyram to honey bees was 3.37 mg/L, and 3, 30 and 300 ug/L
354 were used for the 14 day chronic test, which covered the minimum to maximum
355 residual doses that reported in the reference. The highest concentration in our study
356 was higher than the environmental detection concentration, which was better for
357 toxicological analysis. Our results found nitenpyram decreased the survival and food
358 consumption of honey bees at the sub-lethal concentrations in our study. Therefore, it
359 is recommended to avoid the use of nitenpyram during the flowering or nectar flow of
360 honey bearing plants, or to use products with lower risk to bees as far as possible.

361 It has been well established that the intestinal microbes are essential to governing
362 functions critical to host health, and perturbing it can induce detrimental effects. In
363 honey bees, the gut microbiota is composed of a relatively simple, but highly
364 conservative bacterial community, dominated by the phyla Proteobacteria, Firmicutes,
365 and Actinobacteria (Martinson, 2012). Being consistent with previous study, our
366 results found that Proteobacteria (69.95%), Firmicutes (29.49%) and Actinobacteria
367 (0.51%) were the most abundant at phyla. Although the use of sequencing of 16S
368 rRNA can introduce bias by amplifying species unequally, the consistent community
369 at phyla indicated the bias introduced by the sequencing did not affect the result in our
370 study. Nitenpyram did not eliminate colonization by any core member at the phylum
371 level. However, at the class level, the abundance of Betaproteobacteria, Bacilli and
372 Alphaproteobacteria were increased, while that of Gammaproteobacteria was
373 strikingly decreased in the nitenpyram treated groups. At the genus level, several key
374 gut microbiota were significantly altered. Firstly, it should be noted that, Gilliamella,
375 one of the most dominant members of the honey bee, was almost entirely absent in the

376 300 µg/L nitenpyram-treatment group. LEFSe analysis identified that, the core strains
377 of *Gilliamella*, *Gilliamella apicola*, were reduced significantly in nitenpyram-treated
378 bees. Several lines of evidence suggested that honey bee gut microbiota had the
379 function of metabolizing diverse compounds (Philipp et al., 2013). Philipp et al.
380 demonstrated that *Gilliamella* was capable of degrading pectin which is a compound
381 of the pollen cell wall and ferment mannose, arabinose, xylose and rhamnose (Philipp
382 et al., 2013). By metabolizing such sugars, *Gilliamella* could be critical for the
383 detoxification of food components (Philipp et al., 2013). Genomic and metabolic
384 studies on bee gut core species indicated that *Gilliamella apicola* harbored the
385 capabilities to digest and metabolize a diverse array of plant-produced carbohydrates
386 (Raymann and Moran, 2018). Besides, it has become evident that gut is a natural
387 anti-inflammatory barrier and some of the bacterial changed were tightly related to
388 disease and immunity (Schiffrin and Donnet-Hughes, 2011). A previous study
389 demonstrated that infection by the gut parasite *Critchidia* was negatively associated
390 with abundance of *Gilliamella* in *Bombus*, indicating that *Gilliamella* was associated
391 with protection against pathogens and immunity (Cariveau et al., 2014). Kwong et.al
392 also proved bees that were initially inoculated with *Gilliamella apicola* wkB7 showed
393 significantly higher post-infection survival rates when compared with bees lacking gut
394 symbionts (Kwong et al., 2017). Therefore, the loss of *Gilliamella* and *Gilliamella*
395 *apicola* might be one of important reasons for affecting the carbohydrates metabolism
396 and immunity in nitenpyram-exposed bees in our study. Furthermore, in the current
397 study, the relative abundance of another important member of Gammaproteobacteria,
398 *Frischella perrara*, was significantly increased in nitenpyram-exposed bee gut. Engel
399 et al. demonstrated that *Frischella perrara* triggered a morphological change of the
400 epithelial surface, potentially due to a host immune response, showing that *Frischella*

401 *perrara* possessed a potential role in immunity (Engel et al., 2015). Thus, the
402 alteration of *Frischella perrara* relative abundance might also be involved in the
403 abnormality of immune adjustment induced by nitenpyram. Additionally, the relative
404 abundance of *Bifidobacterium* were increased in nitenpyram-treated bees.
405 *Bifidobacterium* were proved to possess capabilities to digest and metabolize
406 carbohydrates (Raymann and Moran, 2018). Hence, we speculated that the increased
407 *Bifidobacterium* might be a regulation of dynamic balance of intestinal function to
408 compensate for the loss of function in the absence of *Gilliamella*. Overall, these
409 results indicated that nitenpyram induced gut microbiota dysbiosis in honey bees and
410 might further cause disorders of bee intestinal immune system and metabolism.

411 In recent years, the gut microbiota has been recognized to affect host biological
412 processes through influencing the host related gene expression. Therefore, we
413 performed high-throughput RNA-Seq transcriptomic analysis to further elucidate gut
414 genes involved in diverse biological processes regulated by nitenpyram. In the current
415 study, we confirmed that several key genes related to glycolysis and lipid metabolism
416 in the gut were altered by nitenpyram exposure, suggesting metabolism processes
417 were disrupted in nitenpyram-treated bees. For example, the expression of the gene
418 encoding the phosphoenolpyruvate carboxykinase (PEPCK) (Gene ID 412843), a key
419 enzyme in the synthesis of glucose (Lv et al., 2017), was reduced in bees exposed to
420 nitenpyram. While the expression of maltase A3, an enzyme which hydrolyzes
421 α-D-glucosides (Moody and Purdy, 1971), was significantly up-regulated in our study.
422 Additionally, we found the gene expression of apolipophorins (Gene ID 408961),
423 which is involved in lipid transport serving as lipid transport vehicles (Stä...Czek et
424 al., 2017), was significantly down-regulated. Similarly, a member of the fatty acid
425 hydroxylase superfamily, FAXDC2 (Gene ID 727357), which plays a potential role in

426 lipid metabolism, was also inhibited in nitenpyram-treated honey bees (Jin et al.,
427 2016). On the other hand, increasing amounts of convincing evidence now indicate
428 that the housekeeping metabolic enzymes involved in glucose and fatty acid synthesis
429 may play a critical role in the hypothalamic regulation of feeding (Lopez and Lelliott,
430 2010). Therefore, the disordered metabolism caused by nitenpyram might be one of
431 the reasons for the decreased food consumption in bees.

432 It is noteworthy that the expression of cholecystokinin receptor (CCKR)-like
433 (Gene ID 4106542) was significant down-regulated in nitenpyram-treated bees' gut.
434 The cholecystokinin receptor (CCKR) is mainly expressed in the brain and the
435 alimentary tract, regulating the feeding behavior in the way of negative feedback
436 (Dorien et al., 2011). Hence, the decreased gene expression of cholecystokinin
437 receptor (CCKR)-like might be partly involved in regulating the feeding behavior of
438 nitenpyram-treated bees. In addition, a sensitive olfactory system seems to be
439 particularly important in insects for nutrition (detecting food sources). It has been
440 proposed that odorant-binding proteins (OBPs) and olfactory receptors (ORs) are
441 involved in insect odor recognition and chemical communication(Li et al., 2017).
442 Here, both the expression of genes encoding odorant-binding proteins 13 (OBP13)
443 (Gene ID 677674) and odorant receptor 4 (OR4) (Gene ID 100578402) were
444 significant altered in nitenpyram-treated bees, suggesting that the olfactory of
445 nitenpyram-treated bees might be affected, which could cause them to refuse feeding.
446 Overall, these data may be the potential molecular mechanism for the decreased food
447 consumption of nitenpyram-treated bees.

448 Aufauvre et.al demonstrated that chronic exposure to another two neonicotinoid
449 insecticides, fipronil and imidacloprid, could repress the expression of
450 immunity-related genes in honey bee gut (Aufauvre et al., 2014). Similar with the

451 previous research, several genes related to immunity and pathogen defence were also
452 significantly altered in gut of nitenpyram-treated bees in our experiment. The gene
453 expression of a bee venom peptide, secapin (Gene ID 406145), which is an
454 anti-microbial peptide with activity against bacteria and fungi in the innate immune
455 response (Lee et al., 2016), was significantly down-regulated in nitenpyram-treated
456 bees, indicating the innate immune response was repressed after nitenpyram treated.
457 Simultaneously, Toll-like receptor 4 (TLR4) encoding gene (Gene ID 724187), which
458 mediates recognition of lipopolysaccharide (LPS) by the host and is responsible for
459 the early innate immune response (Schurr et al., 2005), was also shown a significant
460 inhibition. Endochitinase is believed to be important in the biochemical defense
461 against potential pathogens (Cheng et al., 2017). While a significant inhibition of the
462 gene encoding endochitinase (Gene ID 551600) was also observed in nitenpyram
463 exposed honey bee gut, as well as a putative defense protein encoding gene (Gene ID
464 726072). It is important to note that most of them showed down-regulation trend,
465 suggesting that nitenpyram had an inhibitory effect on gut immunity of bees.
466 Therefore, considering the altered abundance of several key gut microbiota related to
467 immunity in nitenpyram-treated bees, it can be inferred that nitenpyram disrupted gut
468 microbiota and then repress the gut immunity system, even leading to individual death.
469 In addition, the expressions of several detoxification related genes were also inhibited
470 in nitenpyram-treated honey bee gut. In our study, the gene expression of CYP6a14
471 (Gene ID 724946) was decreased by nitenpyram. Previous studies have shown that
472 cytochrome P450-mediated detoxification contributes to xenobiotics tolerance in
473 many insects (Zhou et al., 2010). Recent study also found the gene of another member
474 of the CYP6AS subfamily, CYP6as5, was reduced in another neonicotinoid
475 thiamethoxam treated honey bees (Shi et al., 2017), indicating that CYP6AS

476 subfamily might be involved in the metabolism process of neonicotinoid insecticides.
477 Several other detoxification related genes were also altered, such as genes encoding
478 esterase E4 (Gene ID 409801) and peroxidase-like (Gene ID 100577249), which
479 suggested that nitenpyram might inhibit the detoxification system in the gut of bees.
480 This may be another one of the reasons that nitenpyram-treated honey bees possessed
481 a lower survival. It should be noted that down regulation of some metabolic enzymes
482 may enable resource to be redirected to up regulation of those responsible for
483 detoxication of exogenous toxicant (Pavek and Dvorak, 2008). In the current study, it
484 was not clear whether the alteration of the expressions of several detoxification
485 related genes were adverse or just adaptive to detoxification of nitenpyram. More
486 proof studies should be carried out in the future.

487 These clustered genes were also grouped into several pathways based on GO and
488 KEGG. The results of GO and KEGG analysis showed that the regulation of many
489 DEGs related to kinds of metabolism processes, which further concerned that
490 nitenpyram mainly affected metabolism in honey bees. This was consistence with our
491 previous finding on gut microbiota. A analogous research was carried out previously,
492 in which the neonicotinoid thiamethoxam regulated many DEGs related to
493 metabolism processes in honey bees (Shi et al., 2017).

494 It should be noted that reduced food consumption might also be expected to
495 change gut microbiota and gene profiles. A previous research reported the effects of
496 starvation on the diversity of gut microbiota of adult desert locusts, and the results
497 showed an increased diversity of Gammaproteobacteria in starved locusts (Dillon et
498 al., 2010). On the contrary, the diversity of Gammaproteobacteria was significantly
499 decreased in nitenpyram-treated bees in our study. Another research investigated how
500 the gut microbiota, digestion and immune activities of shrimp were affected under

501 starvation stress (Dai et al., 2018b). The results showed that the measured immune
502 activities exhibited an increased trend and the structures of gut bacterial communities
503 were more abundant when shrimps were under starvation stress. These observations
504 were not consistent with our results, in which the measured immune activities
505 exhibited a decreased trend and the structures of gut bacterial communities were not
506 more abundant in bees exposed to nitenpyram. If the regulatory mechanisms of
507 reduced food consumption on gut microbiota and gut gene profiles are similar among
508 different species, it could be inferred that reduced food consumption was not the main
509 factor that caused the changes in gut microbiota and the decline of intestinal immunity
510 in our study. Therefore, we speculated it was the nitenpyram exposure but not reduced
511 food consumption that drove the changes in gut microbiota and the decline of
512 intestinal immunity in bees in our study. However, considering the lack of research on
513 the effects of reduced food consumption on bee gut, further confirmation should be
514 carried out in the future.

515 **5. Conclusion**

516 In conclusion, our study revealed that nitenpyram exposure altered the
517 abundance of several key microbiotas which contributed to metabolism and immunity
518 in honey bee gut, with affecting the metabolic homeostasis and immunity of bees. It
519 was further verified by the transcriptomic results in which a lot of genes involved in
520 metabolism and immunity were changed. Therefore, it is possible to hypothesize that
521 disordered metabolism and inhibited immunity caused by alteration of the gut
522 microbiota could directly or indirectly affect the food consumption and health of
523 honey bees after nitenpyram exposure. However, there is lack of direct evidence to
524 support this hypothesis in the present study, and more work should be carried out in
525 the future.

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 529 Grant (No.21707162) and the Agricultural Science and Technology Innovation
 530 Program under Grant (talent training programs, No.180818).

531 **References**

- 532 Alfred, E., Matthias, H., Bernd, S., Wolfgang, T., Ralf, N., 2010. Applied aspects of neonicotinoid uses
 533 in crop protection. Pest Management Science 64, 1099-1105.
- 534 Aufauvre, J., Mismeaucouturier, B., Viguès, B., Texier, C., Delbac, F., Blot, N., 2014. Transcriptome
 535 Analyses of the Honeybee Response to Nosema ceranae and Insecticides. Plos One 9, e91686.
- 536 Axel, D., Eric, L., Minh-Hà, P.D., 2003. Learning performances of honeybees (*Apis mellifera* L) are
 537 differentially affected by imidacloprid according to the season. Pest Management Science 59, 269–278.
- 538 Brandt, A., Gorenflo, A., Siede, R., Meixner, M., Büchler, R., 2016. The neonicotinoids thiacloprid,
 539 imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.).
 540 Journal of Insect Physiology 86, 40-47.
- 541 Cariveau, D.P., Elijah Powell, J., Koch, H., Winfree, R., Moran, N.A., 2014. Variation in gut microbial
 542 communities and its association with pathogen infection in wild bumble bees (*Bombus*). Isme Journal 8,
 543 2369-2379.
- 544 Carla, S., Paula, A., Patrícia, V., 2016. Relationships of *Echium plantagineum* L. bee pollen, dietary
 545 flavonoids and their colonic metabolites with cytochrome P450 enzymes and oxidative stress. Rsc
 546 Advances 6, 6084-6092.
- 547 Cheng, X.X., Zhao, L.H., Klosterman, S.J., Feng, H.J., Feng, Z.L., Wei, F., Shi, Y.Q., Li, Z.F., Zhu,
 548 H.Q., 2017. The endochitinase VDECH from *Verticillium dahliae* inhibits spore germination and
 549 activates plant defense responses. Plant Science 259, 12-23.
- 550 Codling, G., Naggar, Y.A., Giesy, J.P., Robertson, A.J., 2016. Concentrations of neonicotinoid
 551 insecticides in honey, pollen and honey bees (*Apis mellifera* L.) in central Saskatchewan, Canada.
 552 Chemosphere 144, 2321-2328.
- 553 Correia, T.R., Scott, F.B., Verocai, G.G., Souza, C.P., Fernandes, J.I., Melo, R.M.P.S., Vieira, V.P.C.,
 554 Ribeiro, F.A., 2010. Larvicidal efficacy of nitenpyram on the treatment of myiasis caused by
 555 *Cochliomyia hominivorax* (Diptera: Calliphoridae) in dogs. Veterinary Parasitology 173, 169-172.
- 556 Dai, P., Z, Y., S, M., Y, Y., Q, W., C, H., Y, W., Y, L., Q, D., 2018a. The Herbicide Glyphosate
 557 Negatively Affects Midgut Bacterial Communities and Survival of Honey Bee during Larvae Reared in
 558 Vitro. Journal of Agricultural and Food Chemistry 66, 7786-7791.
- 559 Dai, W.F., Zhang, J.J., Qiu, Q.F., Chen, J., Yang, W., Ni, S., Xiong, J.B., 2018b. Starvation stress affects
 560 the interplay among shrimp gut microbiota, digestion and immune activities. Fish & Shellfish
 561 Immunology 80, 191-199.
- 562 Decourtey, A., Devillers, J., 2010. Ecotoxicity of neonicotinoid insecticides to bees. Oxygen Transport
 563 to Tissue XXXIII 683, 85-95.
- 564 Dillon, R.J., Gordon, W., Weightman, A.J., Charnley, A., Keith, 2010. Diversity of gut microbiota
 565 increases with aging and starvation in the desert locust. Antonie Van Leeuwenhoek 97, 69-77.

- 566 Dong, X., Jiang, D., Liu, Q., Han, E., Zhang, X., Guan, X., Wang, K., Qiu, B., 2014. Enhanced
 567 amperometric sensing for direct detection of nitenpyram via synergistic effect of copper nanoparticles
 568 and nitrogen-doped graphene. *Journal of Electroanalytical Chemistry* 734, 25-30.
- 569 Dorien, S., Elnaz Karimian, A., Olivier, C., Jérôme, B., Laurence, L., John, V.C., Guy, S., 2011. The
 570 CCK(-like) receptor in the animal kingdom: functions, evolution and structures. *Peptides* 32, 607-619.
- 571 Engel, P., Bartlett, K.D., Moran, N.A., 2015. The Bacterium *Frischella perrara* Causes Scab Formation
 572 in the Gut of its Honeybee Host. *Mbio* 6, 00193-00115.
- 573 Fredrik, G., Marina, V.-R., Consuelo, R.-G., E., K.O., Deborah, K., Sándor, B., Manuel, S.-V.J., Amit,
 574 K., Metagenomic Detection of Viral Pathogens in Spanish Honeybees: Co-Infection by Aphid Lethal
 575 Paralysis, Israel Acute Paralysis and Lake Sinai Viruses. *Plos One* 8, e57459-.
- 576 Guez, D., Suchail, S., Gauthier, M., Maleszka, R., Belzunces, L.P., 2001. Contrasting effects of
 577 imidacloprid on habituation in 7- and 8-day-old honeybees (*Apis mellifera*). *Neurobiology of Learning & Memory*
 578 76, 183-191.
- 579 Hassani, A.K.E., Dacher, M., Gary, V., Lambin, M., Gauthier, M., Armengaud, C., 2008. Effects of
 580 Sublethal Doses of Acetamiprid and Thiamethoxam on the Behavior of the Honeybee (*Apis mellifera*).
 581 *Archives of Environmental Contamination & Toxicology* 54, 653-661.
- 582 Hirotaka, O., Masahiro, O., Kazuhiko, A., Yoko, K., Shinjiro, H., 2003. Determination of neonicotinoid
 583 pesticide residues in vegetables and fruits with solid phase extraction and liquid chromatography mass
 584 spectrometry. *Journal of Agricultural & Food Chemistry* 51, 2501.
- 585 Hung, K.J., Kingston, J.M., Albrecht, M., Holway, D.A., Kohn, J.R., 2018. The worldwide importance
 586 of honey bees as pollinators in natural habitats. *Proceedings Biological Sciences* 285, 2017-2140.
- 587 Jin, Q., Ren, Y., Wang, M., Suraneni, P.K., Li, D., Crispino, J.D., Fan, J., Huang, Z., 2016. Novel
 588 function of FAXDC2 in megakaryopoiesis. *Blood Cancer Journal* 6, 478.
- 589 Kwong, W.K., Mancenido, A.L., Moran, N.A., 2017. Immune system stimulation by the native gut
 590 microbiota of honey bees. *Royal Society Open Science* 4, 170003.
- 591 Lee, K.S., Kim, B.Y., Yoon, H.J., Choi, Y.S., Jin, B.R., 2016. Secapin, a bee venom peptide, exhibits
 592 anti-fibrinolytic, anti-elastolytic, and anti-microbial activities. *Developmental & Comparative
 593 Immunology* 63, 27-35.
- 594 Li, N., Sun, X., Wang, M.Q., 2017. Expression pattern and ligand-binding properties of
 595 odorant-binding protein 13 from *Monochamus alternatus* hope. *Journal of Applied Entomology* 141.
- 596 Lopez, M., Lelliott, C.P., A, 2010. Hypothalamic fatty acid metabolism: a housekeeping pathway that
 597 regulates food intake. *Bioessays* 29, 248-261.
- 598 Lv, Z., Qiu, L., Wang, W., Liu, Z., Xue, Z., Yu, Z., Song, X., Chen, H., Wang, L., Song, L., 2017. A
 599 GTP-dependent Phosphoenolpyruvate Carboxykinase from *Crassostrea gigas* involved in immune
 600 recognition. *Developmental & Comparative Immunology* 77, 318.
- 601 Martinson, V.G., 2012. Establishment of characteristic gut bacteria during development of the honeybee
 602 worker. *Applied & Environmental Microbiology* 78, 2830-2840.
- 603 Moody, J.R., Purdy, W.C., 1971. The coulometric determination of maltose with maltase. *Analytica
 604 Chimica Acta* 53, 239-247.
- 605 Morrissey, C.A., Mineau, P., Devries, J.H., Sanchez-Bayo, F., Liess, M., Cavallaro, M.C., Liber, K.,
 606 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates:
 607 A review. *Environment International* 74, 291-303.
- 608 Motta, E.V., Raymann, K., Moran, N.A., 2018. Glyphosate perturbs the gut microbiota of honey bees.
 609 *Proceedings of the National Academy of Sciences* 115, 10305-10310.

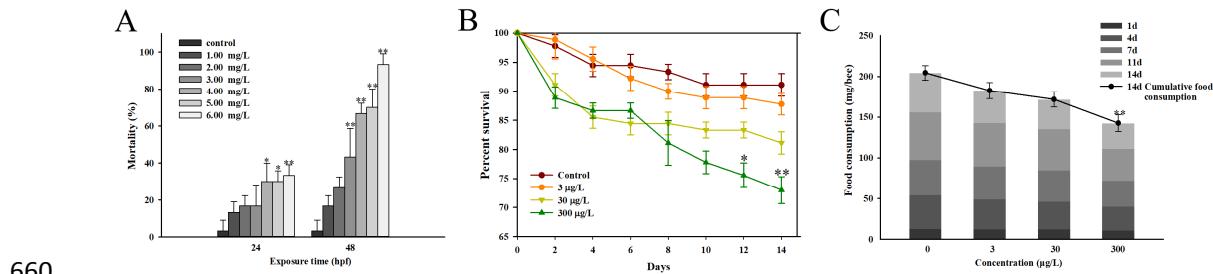
- 610 Pavek, P., Dvorak, Z., 2008. Xenobiotic-Induced Transcriptional Regulation of Xenobiotic
 611 Metabolizing Enzymes of the Cytochrome P450 Superfamily in Human Extrahepatic Tissues. Current
 612 Drug Metabolism 9, 129-143.
- 613 Philipp, E., Martinson, V.G., Moran, N.A., 2013. Functional diversity within the simple gut microbiota
 614 of the honey bee. Proceedings of the National Academy of Sciences of the United States of America
 615 109, 11002-11007.
- 616 Raymann, K., EVS, M., C, G., IM, R., JA, D., NA, M., 2018. Imidacloprid Decreases Honey Bee
 617 Survival Rates but Does Not Affect the Gut Microbiome. Applied & Environmental Microbiology 84,
 618 AEM.00545-00518.
- 619 Raymann, K., Moran, N.A., 2018. The role of the gut microbiome in health and disease of adult honey
 620 bee workers. Current Opinion in Insect Science 26, S2214574517301761.
- 621 Schiffrin, E.J., Donnet-Hughes, A., 2011. Microbiota, Probiotics and Natural Immunity of the Gut.
- 622 Schurr, J.R., Erana, Y., Pat, B., Chad, S., Shellito, J.E., Kolls, J.K., 2005. Central role of toll-like
 623 receptor 4 signaling and host defense in experimental pneumonia caused by Gram-negative bacteria.
 624 Infection & Immunity 73, 532-545.
- 625 Shi, T.F., Wang, Y.F., Liu, F., Qi, L., Yu, L.S., 2017. Sublethal Effects of the Neonicotinoid Insecticide
 626 Thiamethoxam on the Transcriptome of the Honey Bees (Hymenoptera: Apidae). Journal of Economic
 627 Entomology 110, 2283-2289.
- 628 Song, S., Zhang, C., Chen, Z., He, F., Wei, J., Tan, H., Li, X., 2018. Simultaneous determination of
 629 neonicotinoid insecticides and insect growth regulators residues in honey using LC-MS/MS with anion
 630 exchanger-disposable pipette extraction. Journal of Chromatography A 1557, 51.
- 631 Stä...Czek, S., Zdybicka-Barabas, A., Mak, P., Sowa-Jasiå, Ek, A., Kedracka-Krok, S., Jankowska, U.,
 632 Suder, P., Wydrych, J., Grygorczuk, K., Jakubowicz, T., 2017. Studies on localization and protein
 633 ligands of Galleria mellonella apolipophorin III during immune response against different pathogens.
 634 Journal of Insect Physiology 105, 18-27.
- 635 Williams, G.R., Troxler, A., Retschnig, G., Roth, K., Yañez, O., Shutler, D., Neumann, P., Gauthier, L.,
 636 2015. Neonicotinoid pesticides severely affect honey bee queens. Scientific Reports 5, 14621.
- 637 Wu, R.H., Ding, Y., Yan, H.J., Xin, X., Guo, X.Y., Yu, X.Y., Song, W., 2016. Safety evaluation of
 638 neonicotinoid insecticides for several environmental organisms. Jiangsu Agricultural Sciences 44,
 639 295-297.
- 640 Wu, Y.Y., Luo, Q.H., Hou, C.S., Wang, Q., Dai, P.L., Gao, J., Liu, Y.J., Diao, Q.Y., 2017. Sublethal
 641 effects of imidacloprid on targeting muscle and ribosomal protein related genes in the honey bee *Apis*
 642 *mellifera* L. Sci Rep 7, 15943.
- 643 Yang, E.C., Chuang, Y.C., Chen, Y.L., Chang, L.H., 2008. Abnormal foraging behavior induced by
 644 sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). Journal of Economic
 645 Entomology 101, 1743-1748.
- 646 Yang, Y., Ma, S., Yan, Z., Liu, F., Diao, Q., Dai, P., 2019. Effects of three common pesticides on
 647 survival, food consumption and midgut bacterial communities of adult workers *Apis cerana* and *Apis*
 648 *mellifera*. Environ Pollut 249, 860-867.
- 649 Zhang, B., Chen, C., Zhang, H., Feng, L., He, H., Sun, G., 2017. The Toxicity Effects of Abamectin on
 650 Honeybees(*Apis mellifera* L.). Asian Journal of Ecotoxicology.
- 651 Zhou, X., Sheng, C., Li, M., Wan, H., Liu, D., Qiu, X., 2010. Expression responses of nine cytochrome
 652 P450 genes to xenobiotics in the cotton bollworm *Helicoverpa armigera*. Pesticide Biochemistry &
 653 Physiology 97, 209-213.

654 Zhu, L., Mu, X., Wang, K., Chai, T., Yang, Y., Qiu, L., Wang, C., 2015. Cyhalofop-butyl has the
 655 potential to induce developmental toxicity, oxidative stress and apoptosis in early life stage of zebrafish
 656 (*Danio rerio*). Environ Pollut 203, 40-49.

657

658 **Figures:**

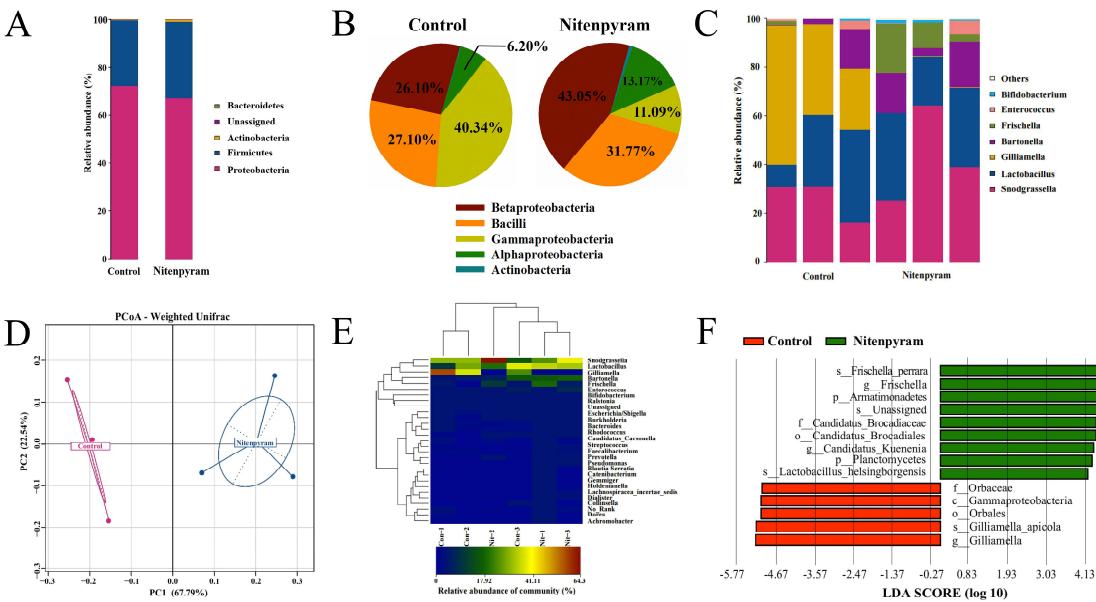
659 **Fig. 1**



660

661 **Fig. 1.** The toxicity effects of honey bees exposed to 300 µg/L nitenpyram
 662 relative to control bees. (A) Acute mortality of adult honey bees after nitenpyram
 663 exposure at 24h and 48h. (B) Survival of honey bees exposed to various nitenpyram
 664 concentrations during newly emerged bees until 14d. (C) The cumulative food
 665 consumption of honey bees exposed to various nitenpyram concentrations during
 666 newly emerged bees until 14d. The asterisks indicate significant differences from the
 667 control group (determined by Dunnett post-hoc comparison, * $p<0.05$; ** $p<0.01$).
 668 Error bars represent the standard deviation.

669 **Fig. 2**

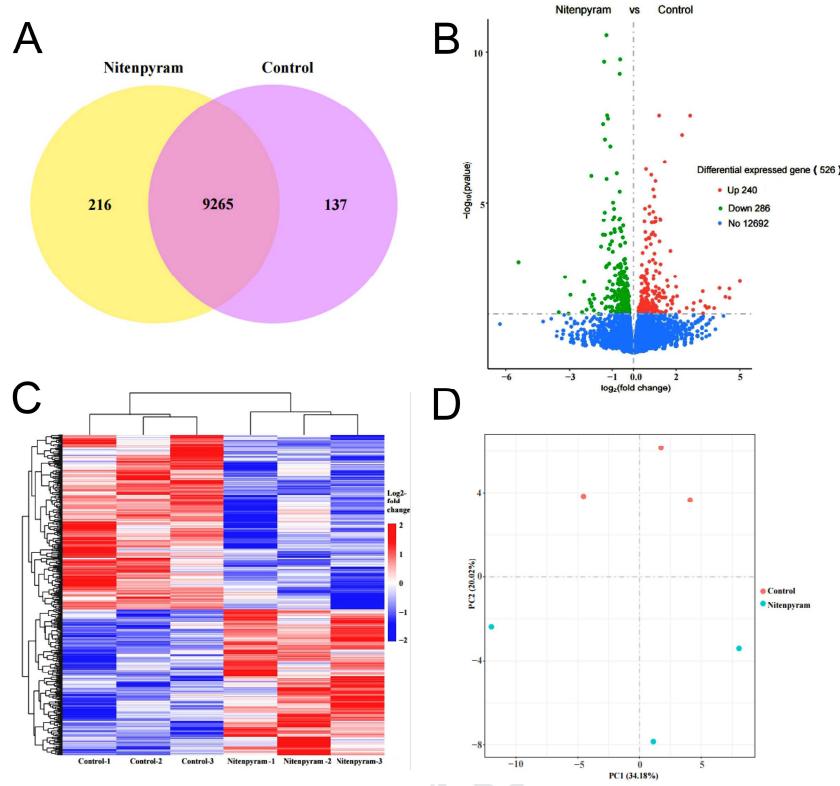


670

671 **Fig. 2.** Effects of nitenpyram exposure on the gut microbiota composition in
 672 honey bees. (A) The composition of gut microbiota at the phylum level in the control
 673 group and 300 μ g/L nitenpyram-treated group. (B) Composition profiles of gut
 674 microbiota at the class level in the control group and 300 μ g/L nitenpyram-treated
 675 group. (C) The percentage of sequences annotated at the genus level in the control
 676 group and 300 μ g/L nitenpyram-treated group. (D) UniFrac principal component
 677 analysis (PCoA) estimates of gut microbiota of the control and 300 μ g/L
 678 nitenpyram-treated groups. ($R^2=0.31$, $P=0.20$) (E) Hierarchical cluster heat map
 679 representation of the top 30 most abundant genera in the control and 300 μ g/L
 680 nitenpyram-treated group. (F) LEFSe analysis illustrating differentially abundant
 681 bacteria among samples with different haze levels. LDA scores (bacteria that obtain a
 682 log LDA score of >4 are ultimately considered) can be interpreted as the degree of
 683 consistent difference in relative abundance among three treatments.

684

685 **Fig. 3**



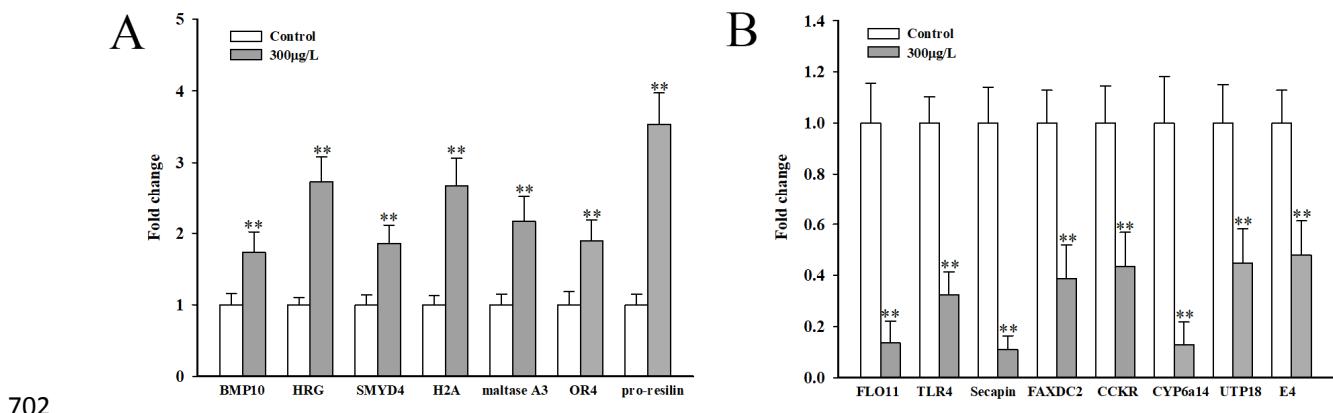
686

687 **Fig. 3.** Differential gene expression between honey bees exposed to 300 $\mu\text{g/L}$
 688 nitenpyram relative to control bees. (A) Venn diagram representing the number of
 689 significantly differentially expressed genes (SDEGs) between nitenpyram and control
 690 bees. The intersection corresponds to genes that were significantly differentially
 691 expressed in both group. (B) Volcano plot of differentially expressed genes in honey
 692 bees exposed 300 $\mu\text{g/L}$ nitenpyram over 14 d. Red points: upregulated genes in
 693 nitenpyram-treated bees; green points: downregulated genes in nitenpyram-treated
 694 bees; blue points: no significant difference. (C) Heatmap showing the log₂-fold
 695 changes of gene expression between each replicate and the average RPKM value for
 696 control bees. (D) Principal component analysis (PCA) of differentially expressed
 697 genes between nitenpyram and control bees.

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700

701 **Fig. 4**

702

703 **Fig. 4.** Real-time quantitative PCR validation of RNA-Seq data on a selection of
 704 fifteen DEG in honey bees exposed to control and 300 µg/L nitenpyram over 14 d. (A)
 705 7 DEG were selected from the most up-regulated genes for qPCR validation (BMP10,
 706 HRG, SMYD4, H2A, maltase A3, OR4 and pro-resilin). (B) 8 DEG were selected
 707 from the most down-regulated genes for qPCR validation (FLO11, TLR4, Secapin,
 708 FAXDC2, CCKR, CYP6a14, UTP18 and E4). Data are means ± SEM. * indicates a
 709 significant difference compared with controls ($P < 0.05$, paired-samples t-test).

710 **Table:**711 **Table 1** The nine significantly enriched pathways, P -value < 0.05 .

Pathway	Pathway ID	Genes number	P -value
Pentose and glucuronate interconversions	ame00040	5	7.35E-05
Drug metabolism - other enzymes	ame00983	4	0.002959
Ascorbate and aldarate metabolism	ame00053	3	0.005602
Glycerophospholipid metabolism	ame00564	6	0.011375
Starch and sucrose metabolism	ame00500	3	0.023208
Sphingolipid metabolism	ame00600	3	0.035083
Tryptophan metabolism	ame00380	3	0.039619
Porphyrin and chlorophyll metabolism	ame00860	3	0.039619
Retinol metabolism	ame00830	2	0.045561

712

Tables:**Table 1** The nine significantly enriched pathways, *P*-value < 0.05.

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Porphyrin and chlorophyll metabolism	ame00860	3	0.039619
Retinol metabolism	ame00830	2	0.045561

Highlights:

1. Nitenpyram decreased the survival and food consumption of honey bees.
2. Nitenpyram disturbed the gut microbiota in honeybees.
3. Nitenpyram affected metabolism and immunity putatively caused by disordered guts microbiota.

Declaration of interests

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: