

High housing density increases stress hormone- or disease-associated fecal microbiota in male Brandt's voles (*Lasiopodomys brandtii*)

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ABSTRACT

Density-dependence is an important mechanism in the population regulation of small mammals. Stressors induced by high-density (e.g., crowding and aggression) can cause physiological and neurological disorders, and are hypothesized to be associated with alterations in gut microbiota, which may in turn reduce the fitness of animals by increasing stress- or disease-associated microbes. In this study, we examined the effects of housing density on the hormone levels, immunity, and composition of gut microbiota in male Brandt's voles (*Lasiopodomys brandtii*) by conducting two specific housing density experiments with or without physical contact between voles. Voles in high density groups exhibited higher serum corticosterone (CORT), serotonin (5-HT), and immunoglobulin G (IgG) levels, as well as higher testosterone (T) levels only in the experiment with physical contact. Meanwhile, high-density treatments induced significant changes in the composition of gut microbiota by increasing disease-associated microbes. The levels of hormones and immunity (i.e., CORT, 5-HT, and IgG) elevated by the high density treatment were significantly correlated with some specific microbes. These results imply that high-density-induced stress may shape the fitness of animals under natural conditions by altering their gut microbiota. Our study provides novel insights into the potential roles of gut microbiota in the density-dependent population regulation of small rodents as well as the potential mechanisms underlying psychological disorders in humans and animals under crowded conditions.

1. Introduction

Populations of animals often fluctuate greatly under field conditions and the influence of both extrinsic and intrinsic factors (Leirs et al., 1997; Zhang, 1996). Density-dependence has long been recognized as a significant mechanism in the population regulation of many small rodent species (Chitty, 1960; Krebs, 1978; Ostfeld and Canham, 1995), such as *Microtus pennsylvanicus* (Ostfeld et al., 1993), *Microtus agrestis* (Ergon et al., 2011), *Rattus norvegicus* (Genaro et al., 2004), and *Mastomys natalensis* (Leirs et al., 1997). Many hypotheses are proposed to demonstrate the population regulation of small mammals, including the genetic regulation hypothesis (Chitty, 1960, 1967), physiological regulation hypothesis (Christian, 1950), and social behavioral regulation hypothesis (Wynne-Edwards, 1962). However, the role of gut microbiota in the population regulation of small mammals has rarely been investigated.

High population densities are known to increase the frequency of aggressive behaviors in small rodents (Nie et al., 2006; Wynne-Edwards, 1962), as well as to induce high social stress and endocrine disorders by increasing corticosterone (CORT) concentrations and decreasing

reproductive hormones; this commonly results in population crashes (Christian, 1950). High population densities accelerate social conflict in individuals by activating the hypothalamic-pituitary-adrenal (HPA) axis and increasing social stress in individuals. Various stressors can affect both social interaction and stress hormones (Lee et al., 2018). Serotonin (5-HT), Testosterone (T), and CORT are crucial for social aggression (Montoya et al., 2012). Both social isolation (Pan et al., 2014) and crowding (Brown and Grunberg, 1995; Palanza et al., 2001) can cause social stress in rodents, affecting central dopamine and 5-HT transmission, and cognitive abilities and affective functions (Arnsten, 2009). The levels of plasma CORT in DBA/2J mice and rhesus macaques (*Macaca mulatta*) are shown to increase under crowded conditions (Lee et al., 2018). Stress-induced increases in glucocorticoids levels are recognized to perform function of immune inhibition and anti-inflammatory reactions in hosts (Bellavance and Rivest, 2014), which reduce defense against parasites and pathogens (Mugabo et al., 2015). High population densities could also increase the risk of pathogen transmission between hosts, and Wilson et al. found that crowded individuals may invest more resources to defend against pathogen infection (Wilson and Reeson, 1998).

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The gut microbes of mammals are essential for their hosts to digest food, maintain health and adapt to the changing environments (Clemente et al., 2012). Gut microbes have increasingly become recognized as crucial regulators to mediate social stress and neuroinflammation (Cryan and Dinan, 2015; Dinan and Cryan, 2012; El Aidy et al., 2014, 2015; Moloney et al., 2014; Sampson and Mazmanian, 2015). Stress plays a crucial role in irritable bowel syndrome (IBS) (Dinan et al., 2010; Elsenbruch, 2011; Mayer, 2000). Stress induced by psychological factors may damage the gut lining by increasing gastrointestinal permeability through the translocation of lipopolysaccharide (LPS) from gram-negative bacteria (Garate et al., 2011). There is strong evidence that there exists a direct link between cytokines and HPA axis activity (Bellavance and Rivest, 2012, 2014; Mastorakos and Ilias, 2000, 2006; Mulla and Buckingham, 1999; Savastano et al., 1994). Psychological stress from chronic social defeat (Bharwani et al., 2016), social disruption (Bailey et al., 2011), and maternal separation (De Palma et al., 2015), can alter gastrointestinal microbiota composition, which could result in disorders such as IBS (Jeffery et al., 2012; Shankar et al., 2015). Moreover, innate and adaptive immunity could be mediated by gastrointestinal microbiota, when the host is faced with infection, inflammation, and autoimmunity (Cassel et al., 2008; El Aidy et al., 2014; Kamada et al., 2013; Mazmanian et al., 2005; Round and Mazmanian, 2009).

High population densities could induce social stress through both crowding and aggression. It may act as a significant physiological and psychological stressor, which compromises the intestinal barrier and increases gastrointestinal permeability with microbiota translocation (Garate et al., 2011). We hypothesized that high housing density could increase the levels of hormones and immunity, and the relative abundance of stress- or disease-associated gut microbiota. The density experiment with physical contact includes stress caused by aggression and crowding, such as space restrictions and odor and sound disruptions, while the density experiment without physical contact only includes stress caused by crowding.

This study aims to test the above hypothesis by using male Brandt's voles (*Lasiopodomys brandtii*) in different housing density groups. The Brandt's voles are distributed in the grasslands of the Inner Mongolia Autonomous Region, and the southeast Baikal region of Russia. They are typical social animals, living in established family groups (Zhong et al., 1999). Their population fluctuates irregularly across the years (Zhang et al., 2003), and population outbreaks would cause huge damage to the grasslands (Zhong et al., 1999). Our previous study indicated that sheep grazing significantly reduces the gut microbiota diversity of Brandt's voles by altering their diet composition (Li et al., 2019). The focus of this study is to examine the effects of high density-induced stress on the gut microbial community and levels of hormones and immunity of voles.

2. Materials and methods

2.1. Experimental animals

Adult Brandt's voles were obtained from a laboratory colony. At the time of weaning (aged three weeks), the voles were housed with siblings of the same sex in groups of three or four and kept on a normal 14:10 h light–dark cycle (lights on from 06:00 to 20:00 h) at room temperature (23 ± 1 °C). Voles had ad libitum access to water and standard rabbit food (47% carbohydrate, 18% protein, 3% fat, and 12% fiber; Beijing KeAo Bioscience Co., Beijing, China). All animal handling or raising procedures were performed in accordance with the Animal Care and Use Committee of the Institute of Zoology, CAS.

2.2. Density experiment without physical contact

High-density-induced stress is caused by crowding (space restrictions, odor and sound disruptions, etc.) and aggressive behaviors from direct contact. A density experiment without physical contact was designed to test the density-induced crowding effects on the gut microbes of voles by

excluding the direct aggression of the animals. A total of 45 male voles (aged eight weeks) were housed individually in 45 metallic braid cages ($24 \times 11.5 \times 10$ cm). Voles were divided into three treatment groups: low crowding, medium crowding, and high crowding. The low-crowding group (L_C) comprised two cages (four replicates) containing one vole each, placed close together at a distance of 0.8 cm (not allowing voles to directly contact each other), giving each vole only one neighbor. The medium-crowding group (M_C) had four cages (three replicates) containing one vole each, placed 0.8 cm apart in a 2×2 grid on the ground, giving each vole three neighbors. The high-crowding group (H_C) had 25 cages containing one vole each, placed 0.8 cm apart in a 5×5 grid on the ground; this design allowed each of the nine voles in the interior of the grid to have eight neighbors. The H_C group had no independent replicates for minimizing the usage of animals, but each of the nine voles in the interior of the grid could be regarded as nine replicates. The cages were made of stainless-steel mesh with small holes (1.2×1 cm), which allowed voles to have olfactory, visual, and vocal communications without physical contact, reflecting a pure crowding effect. The experiment lasted for four weeks, and the animals were housed under the same conditions described above. Two voles in the L_C group were excluded for analysis because they were not well adapted to the experimental environments; they continuously chewed the metal wires of their nest box, trying to escape from the adjacent box. Moreover, three voles in the medium-crowding group escaped from their cages and were thus excluded from the analysis. Therefore, the sample sizes for the L_C , M_C , and H_C treatment groups were six, nine, and nine voles, respectively.

2.3. Density experiment with physical contact

A density experiment with physical contact was designed to test density-induced effects, including both crowding and direct agonistic behaviors between voles. A total of 56 male voles (aged eight weeks) were divided into three treatment groups: low density, medium density, and high density. The low-density group (L_D) comprised two voles in one plastic standard cage ($48 \times 25 \times 20$ cm), with eight replicates. The medium-density group (M_D) had four voles in one cage, with four replicates. The high-density group (H_D) had eight voles in one cage, with three replicates. The experiment lasted for four weeks and voles were housed under the same conditions described above. During the experiment, a few voles died from injuries sustained in fights with other voles. In this case, we released new voles into the cages to maintain the density stress during the experiment. By the end of the experiment, twelve voles in the L_D , thirteen voles in the M_D , and eight voles in the H_D groups survived and experienced density-induced stress for four weeks. We selected eight voles from each density group (randomly for the L_D and M_D groups) for later data analysis.

2.4. Sample collection

All voles were weighed at 08:00 h on days 0 and 28 using an electronic balance (Sartorius Model BL 1500, precision 0.1 g). All voles were sacrificed with an intraperitoneal injection of 0.6% sodium pentobarbital (60 mg/kg) at the end of the experiment. Fresh blood and fecal samples for one vole were collected within 2 min. Trunk blood was immediately collected with a 1.5 mL microtube, stood at room temperature (23 ± 1 °C) for 2 h, and centrifuged at $1000 \times g$ for 20 min. All serum samples were frozen immediately at -80 °C. Feces were removed from the colon, frozen immediately on dry ice, and stored at -80 °C. Fresh blood and fecal samples were collected between 08:00 and 10:00 h.

2.5. Measures of CORT, 5-HT, T, and IgG in serum

The serum levels of CORT, 5-HT, T, and IgG in voles were assessed using an enzyme-linked immunosorbent assay (ELISA) kit. All serum levels were determined using commercial vole kits (Jianglai Bio.) and

calculated from a standard curve. The limit of detection for the CORT ELISA kit ranged between 15 ng/mL and 480 ng/mL, and the limit of quantitation was < 1.0 ng/mL. The limit of detection for the 5-HT ELISA kit ranged between 5 ng/mL and 160 ng/mL, and the limit of quantitation was < 1.0 ng/mL. The limit of detection for the T ELISA kit ranged between 25 pg/mL and 800 pg/mL, and the limit of quantitation was < 1.0 pg/mL. The limit of detection for the IgG ELISA kit ranged between 0.5 mg/mL and 16 mg/mL, and the limit of quantitation was < 0.1 mg/mL. The coefficients of variation of intra- and inter-assays were < 10% and 15%, respectively for CORT, 5-HT, T, and IgG.

Fifty microliters of the standard sample and serum sample were added to a 96-well plate, and 100 μ L of horseradish-peroxidase (HRP) conjugate reagent was added. The plate was then covered with the sealing tape and incubated for 1 h at 37 $^{\circ}$ C. After incubation, the plate was manually washed five times with 350 μ L of 1 \times Wash Buffer, and then solutions A and B for Chromogen Substrate were added. After 15 min of incubation at 37 $^{\circ}$ C in the dark, 50 μ L of stop solution was added. After stopping the reactions, the plate was immediately read on a microplate reader (BioTek Instruments, USA) at a wavelength of 450 nm. This assay was performed at room temperature.

2.6. DNA extraction, evaluation, and amplification

Total genomic DNA of microbiota within the colon feces was extracted using cetyltrimethylammonium bromide (CTAB), a phenol chloroform mixture (isoamyl alcohol:chloroform:phenol = 1:24:25). The DNA concentration and purity were detected on 1% agarose gels. DNA concentrations were adjusted to 1 ng/ μ L using sterile water. Then, 16S ribosomal RNA (rRNA) genes were amplified using specific primers with adapter sequences. Primers were set corresponding to the forward primer 341F (5'-CCTAYGGGRBGCASCAG-3') and reverse primer 806R (5'-GGA CTACNNGGGTATCTAAT-3'), targeting the V3–V4 hypervariable 16S rRNA gene region (Dunphy-Doherty et al., 2018; Zhang et al., 2018).

The PCR reaction included 30 μ L reaction solutions with 15 μ L of 2 \times Phusion[®] High-Fidelity PCR Master Mix (New England Biolabs), 3 μ L of primers (2 μ M), and 10 μ L of template DNA (1 ng/ μ L). Thermal cycling included initial denaturation at 98 $^{\circ}$ C for 60 s, followed by 30 cycles (98 $^{\circ}$ C for 10 s, 50 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s), and followed by a final extension at 72 $^{\circ}$ C for 5 min. Equal volume of 1 \times loading buffer (contained SYBR Green) and PCR products were mixed, and then were detected using the electrophoresis on a 2% agarose gel with 1 \times TAE. The GeneJET Gel Extraction Kit (Thermo Scientific) was used to purify the mixed PCR products. All sequencing libraries were constructed using NEB Next[®] Ultra[™] DNA Library Prep Kit for Illumina (NEB, USA), and index codes were added. All libraries were sequenced on an Illumina HiSeq 2500 platform, and then 250 bp paired-end reads were generated.

2.7. 16S rRNA gene amplicon sequencing analysis

Colon fecal samples from individual animals were analyzed separately. All analyses of 16S rRNA gene sequences were performed using Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) software (Caporaso et al., 2010). Paired-end reads from clean data were merged using FLASH (Magoc and Salzberg, 2011). Chimeric sequences were identified and removed using USEARCH, version 10.0.240 (Edgar, 2010). We randomly selected 60,000 (the experiment without physical contact) or 30,000 (the experiment with physical contact) sequences per sample for our analysis to avoid the effect of sequencing depth. Sequences were assigned to operational taxonomic units (OTUs) at a 97% identity threshold referencing the Greengenes database 13_8 (DeSantis et al., 2006). Representative sequences were chosen for each OTU to annotate taxonomic information using the Ribosomal Database Project (RDP) (Cole et al., 2009). We built rarefaction curves with the phylogenetic diversity index, observed species, Shannon index, and Chao1 index. We also calculated the alpha diversity with these four indices. The difference in beta diversity based on the Bray–Curtis

dissimilarity matrix at the OTU level was assessed by the constrained principal coordinate analysis (CPCoA) (Li et al., 2019).

2.8. Statistical analysis

Data analyses were performed using R-3.6.3 software. Data are plotted in the figures as mean \pm SEM. The differences in four serum indicators and alpha-diversity indices between different density groups were assessed using a linear mixed model (LMM) with cages as a random factor (Martínez-Núñez et al., 2019). Significant differences in beta diversity between different groups were evaluated by permutational multivariate analysis of variance (PERMANOVA), which employed the adonis function in the R package vegan with 999 permutations. Statistical analyses employing linear discriminant analysis (LDA) effect size (LefSe) were used to show a normalized relative abundance matrix of microbial communities on the Galaxy platform (<http://huttenhower.sph.harvard.edu/galaxy>). LDA, combined with the Kruskal-Wallis and Wilcoxon sum-rank test was performed in the LefSe analysis (Segata et al., 2011). The correlations between hormones and microbes (at the genus level) were assessed with Pearson's correlation. A probability value of $P < .05$ indicated significant differences. Effect sizes were assessed by Eta-squared (η^2) and Cohen's d .

3. Results

3.1. Effects of density on serum hormones and immunity

The random effect of cages was not significant for serum hormones and immunity (all $P > .05$). In the density experiment without physical contact, linear mixed model analysis revealed that density had a significant effect on the serum concentrations of CORT (LMM, $F_{(2,13)} = 9.377$, $P = .003$, $\eta^2 = 0.20$), 5-HT (LMM, $F_{(2,13)} = 4.053$, $P = .043$, $\eta^2 = 0.018$), and IgG (LMM, $F_{(2,13)} = 4.500$, $P = .033$, $\eta^2 = 0.046$). The serum concentration of CORT in the H_C group was significantly higher than that in the L_C and M_C groups (LMM, $t = 3.6$, $P = .003$, Cohen's $d = 1.90$; $t = 3.7$, $P = .002$, Cohen's $d = 1.74$; Fig. 1A). The concentration of 5-HT in the H_C group was significantly higher than that in the L_C group (LMM, $t = 2.8$, $P = .015$, Cohen's $d = 1.48$; Fig. 1B), while the concentration of IgG in the H_C group was significantly higher than that in the L_C group (LMM, $t = 2.9$, $P = .01$, Cohen's $d = 1.53$; Fig. 1D). There were no significant differences in serum T concentrations among the three treatment groups (LMM, $F_{(2,13)} = 1.111$, $P = .358$, $\eta^2 = 0.083$; Fig. 1C).

In the treatment groups with physical contact, LMM analysis showed that the density significantly affected the levels of CORT (LMM, $F_{(2,18)} = 3.510$, $P = .05$, $\eta^2 = 0.11$), 5-HT (LMM, $F_{(2,18)} = 4.305$, $P = .03$, $\eta^2 = 0.0003$), T (LMM, $F_{(2,18)} = 7.008$, $P = .006$, $\eta^2 = 0.17$), and IgG (LMM, $F_{(2,18)} = 10.523$, $P = .0009$, $\eta^2 = 0.27$). There was a significant difference in the serum concentration of CORT between the M_D and L_D groups (LMM, $t = -2.6$, $P = .02$, Cohen's $d = -1.3$; Fig. 1E). The concentration of 5-HT in the H_D group was significantly higher than that of the L_D group (LMM, $t = 2.9$, $P = .009$, Cohen's $d = 1.45$; Fig. 1F). The concentrations of IgG in the H_D and M_D groups were significantly higher than those of the L_D group (LMM, $t = 3.7$, $P = .002$, Cohen's $d = 1.85$; $t = -4.2$, $P = .0005$, Cohen's $d = -2.1$; Fig. 1H). The serum T concentrations in the H_D and M_D groups were significantly higher than that in the L_D group (LMM, $t = 3.1$, $P = .006$, Cohen's $d = 1.55$; $t = -3.4$, $P = .003$, Cohen's $d = -1.7$; Fig. 1G).

3.2. Effects of density on microbiota diversity

The random effect of cages was not significant in the Chao1 index, phylogenetic diversity index, and observed species, except for the Shannon index of density experiment without physical contact (all $P > .05$). For the alpha diversity analysis, there were no significant differences in alpha diversity indices between the three treatment

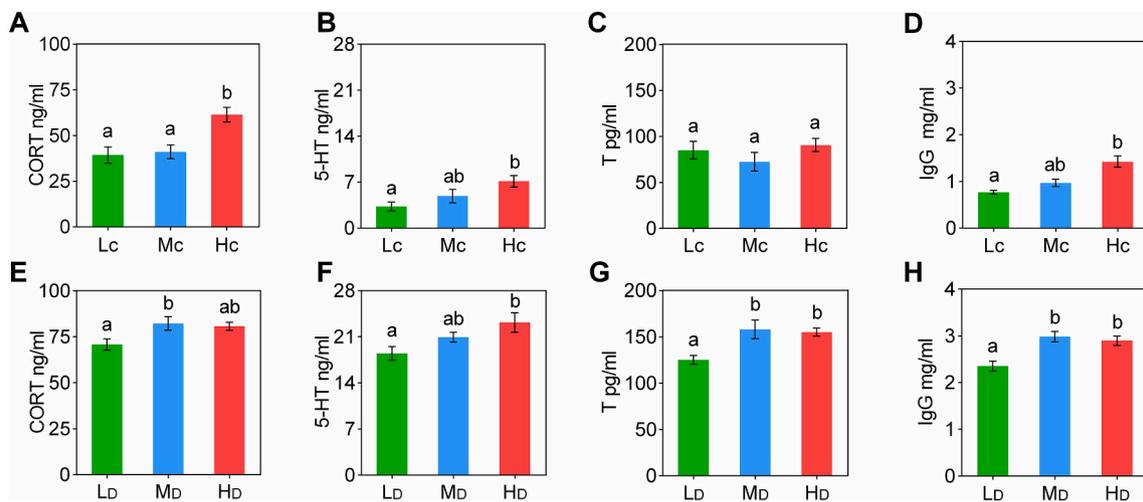


Fig. 1. Effects of laboratory density treatments without (A–D) and with (E–H) physical contact on the concentrations of serum corticosterone (CORT), serotonin (5-HT), testosterone (T), and immunoglobulin G (IgG) in voles. Data are shown as mean ± SEM per group. Variables were analyzed using a linear mixed model (LMM) to assess treatment effects. Bars labeled with different letters are significantly different ($P < .05$). L_C, low-crowding group without physical contact (2 cages/group); M_C, medium-crowding group without physical contact (4 cages/group); H_C, high-crowding group without physical contact (25 cages/group); L_D, low-density group with physical contact (2 voles/cage); M_D, medium-density group with physical contact (4 voles/cage); H_D, high-density group with physical contact (8 voles/cage).

groups in the density experiment without physical contact (all $P > .05$; Fig. S1), but linear mixed model analysis showed that density with physical contact significantly affected the phylogenetic diversity index (LMM, $F_{(2,18)} = 4.380$, $P = .028$, $\eta^2 = 0.13$). The diversity of the phylogenetic diversity index of the H_D group was significantly higher than that of the L_D and M_D groups (LMM, $t = 2.498$, $P = .022$, *Cohen's d* = 1.25; LMM, $t = 2.673$, $P = .016$, *Cohen's d* = 1.34; Fig. S1H).

For the beta diversity analysis, the PERMANOVA revealed that density without physical contact had a significant effect on the gut microbiota community dissimilarity among the three treatment groups (adonis permutation test, $F = 2.670$, $P = .001$, $\eta^2 = 0.21$), with significant discrimination of the bacterial microbiota between them (L_C vs. M_C, $F = 1.989$, $P = .001$, *Cohen's d* = 0.78; L_C vs. H_C, $F = 2.942$, $P = .001$, *Cohen's d* = 0.95; M_C vs. H_C, $F = 3.044$, $P = .001$, *Cohen's d* = 0.87; 14.8% of variance explained; Fig. 2A). Similarly, the PERMANOVA results also suggested that there was an apparent discrimination of the bacterial microbiota among different density groups with physical contact (adonis permutation test, $F = 1.813$, $P = .001$, $\eta^2 = 0.15$; L_D vs. M_D, $F = 1.246$, $P = .181$, *Cohen's d* = 0.60; L_D vs. H_D, $F = 2.224$, $P = .001$, *Cohen's d* = 0.80; M_D vs. H_D, $F = 2.018$, $P = .004$, *Cohen's d* = 0.76; 11.9% of variance explained; Fig. 2B).

3.3. Effects of density on gut microbiota composition

In the density experiment without physical contact, the LefSe results revealed that there were significant differences in the gut microbial community compositions between the L_C, M_C, and H_C groups (Fig. 3A–C). At the phylum level, TM7, Spirochaetes, Proteobacteria, and Firmicutes were enriched in the higher crowding group, whereas Bacteroidetes, Lentisphaerae, and Cyanobacteria were decreased. At the family level, eight families (Christensenellaceae, Desulfovibrionaceae, Prevotellaceae, Spirochaetaceae, Streptococcaceae, F16, Veillonellaceae, and Enterobacteriaceae) were enriched in higher crowding group, whereas the relative abundances of thirteen families (Alteromonadaceae, Rikenellaceae, Halomonadaceae, Odoribacteraceae, Victivallaceae, S24_7, Microbacteriaceae, Pseudomonadaceae, Nocaydiaceae, Erythrobacteraceae, Gordoniaceae, Flavobacteriaceae, and Paraprevotellaceae) were decreased (Table 1). At the genus level, eleven genera (*Faecalibacterium*, *Oscillospira*, *Treponema*, *Dorea*, *Ruminococcus*, *Desulfovibrio*, *Anaerofilum*, *Escherichia*, *Streptococcus*, *Prevotella*, and *Roseburia*) were enriched in higher crowding group, whereas the relative abundances of seventeen genera (*Odoribacter*,

Clostridium, *Halomonas*, *Alteromonas*, *Microbacterium*, *Jeotgalicoccus*, *Erythrobacter*, *Sufflavibacter*, *Pseudomonas*, *Haererehalobacter*, *Victivallis*, *Anaerospira*, *Leeuwenhoekiella*, *Rhodococcus*, *Rikenella*, *Gordonia*, and CF231) were decreased (Table 2).

In the density experiment with physical contact, LefSe results revealed that there were significant differences in the gut microbial community compositions between the L_D, M_D, and H_D groups (Fig. 3D–F). At the phylum level, TM7 and Spirochaetes were decreased in higher density group. At the family level, four families (Peptococcaceae, Ruminococcaceae, Bacteroidaceae, and Erysipelotrichaceae) were enriched in higher density group, whereas the relative abundances of five families (Rikenellaceae, Odoribacteraceae, Spirochaetaceae, F16, and Anaeroplasmataceae) were decreased (Table 1). At the genus level, seven genera (*Faecalibacterium*, *Oscillospira*, *Ruminococcus*, *Anaerofilum*, *Bacteroides*, *Sporobacter*, and *Allobaculum*) were enriched in higher density group, whereas the relative abundances of five genera (*Treponema*, *Odoribacter*, *Clostridium*, *Anaeroplasma*, and *Christensenella*) were decreased (Table 2).

3.4. Correlations between relative abundance of microbes and levels of serum hormones

Table 2 showed the significant correlations between the relative abundance of microbes (at the genus level) and levels of serum hormones and immunity in the density experiments with and without physical contact. In the density experiment without physical contact, the CORT concentration was significantly and positively correlated with the relative abundance of the genera *Oscillospira* ($r = 0.47$, $P = .022$) and *Treponema* ($r = 0.52$, $P = .0091$), and negatively correlated with the genus *Microbacterium* ($r = -0.48$, $P = .018$). The 5-HT concentration was significantly and positively correlated with the relative abundance of the genera *Oscillospira* ($r = 0.50$, $P = .014$), *Treponema* ($r = 0.46$, $P = .025$), *Faecalibacterium* ($r = 0.43$, $P = .034$), and *Desulfovibrio* ($r = 0.43$, $P = .038$). The IgG concentration was significantly and positively correlated with the relative abundance of the genera *Oscillospira* ($r = 0.51$, $P = .011$), *Faecalibacterium* ($r = 0.67$, $P = .00040$), *Desulfovibrio* ($r = 0.52$, $P = .0086$), *Dorea* ($r = 0.52$, $P = .0095$), and *Streptococcus* ($r = 0.41$, $P = .045$), and negatively correlated with the genus *Victivallis* ($r = -0.41$, $P = .045$).

In the density experiment with physical contact, the 5-HT concentration was significantly and positively correlated with the relative abundance of the genera *Oscillospira* ($r = 0.58$, $P = .0030$) and

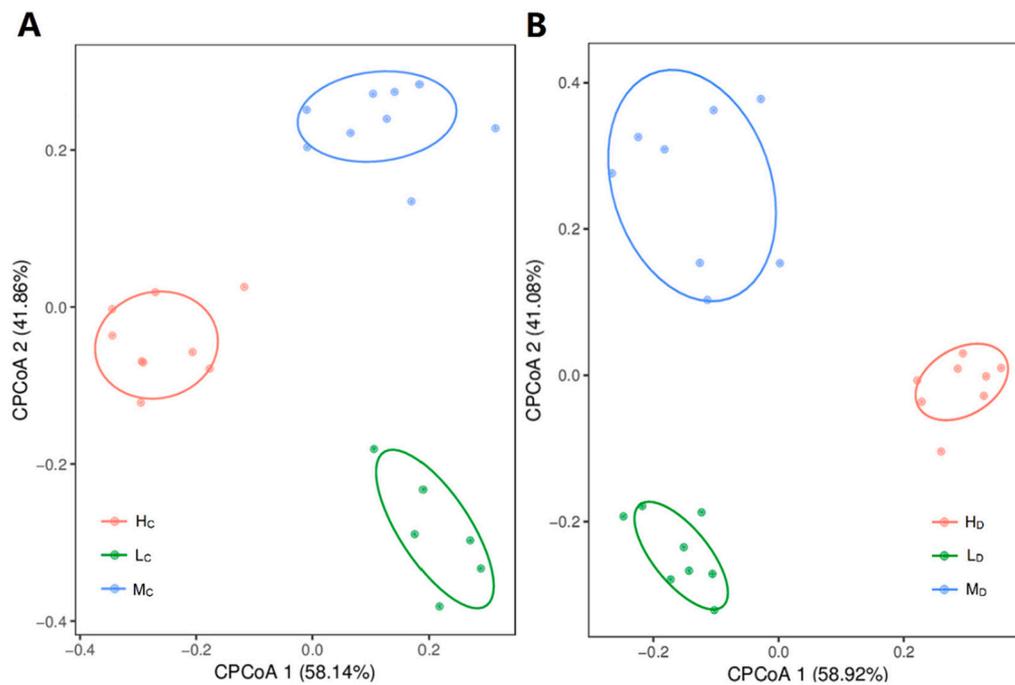


Fig. 2. Beta diversity comparisons of the gut microbiota of colon fecal samples from different treatment groups in laboratory density treatment without (A) and with (B) physical contact. The first two axes are shown with constrained principal coordinate analysis (CPCoA) based on the Bray–Curtis dissimilarity matrix at the OTU level. The plot shows a significant discrimination among the corresponding three groups ($P < .05$). L_C, low-crowding group without physical contact (2 cages/group); M_C, medium-crowding group without physical contact (4 cages/group); H_C, high-crowding group without physical contact (25 cages/group); L_D, low-density group with physical contact (2 voles/cage); M_D, medium-density group with physical contact (4 voles/cage); H_D, high-density group with physical contact (8 voles/cage).

Bacteroides ($r = 0.50$, $P = .013$), the IgG concentration was significantly and positively correlated with the relative abundance of the genus *Sporobacter* ($r = 0.42$, $P = .039$), and the T concentration was significantly and negatively correlated with the relative abundance of the genus *Treponema* ($r = -0.51$, $P = .012$).

4. Discussion

High population density could act as a stressor owing to the increased crowding and aggressive effects, which may affect stress hormones, immunity, and gut microbe composition. In this study, we found that density treatments both with and without physical contact result in remarkable alterations in the gut microbiota at the phylum, family, and genus levels (particularly three genera: *Faecalibacterium*, *Oscillospira*, and *Treponema*) (Tables 1 and 2). High density treatments in the two experiments significantly increase levels of CORT, 5-HT, and IgG, and/or T. Furthermore, these hormones and immunity levels are significantly associated with relative abundance of specific microbes from several genera such as *Faecalibacterium*, *Oscillospira*, *Treponema*, *Dorea*, *Desulfovibrio*, *Bacteroides*, *Microbacterium*, *Sporobacter*, *Streptococcus*, and *Victiallis* (Table 2).

4.1. Impact on gut microbiota

Many factors, including stress, diet, medication, and infection, can alter gut microbiota (Borre et al., 2014; Carmody et al., 2015; David et al., 2014; Kohl et al., 2018). The stress and HPA axis could influence the gut microbiota composition (Sudo et al., 2004). Stress-induced cortisol could change the permeability of the intestinal and barrier function, and alter the composition of the gut microbiota (Cryan and Dinan, 2012). Currently, gut microbiota studies on wild animals are mainly limited to the evolutionary aspects (Ley et al., 2008; Muegge et al., 2011; Xu et al., 2019). Their ecological roles, especially in relation to the population regulation of animals, are rarely investigated. In this study, the housing densities significantly alter the composition of gut microbiota in Brandt's voles, supporting our hypothesis that high density could increase levels of hormones and immunity, and the relative abundance of stress- or disease-associated gut microbiota.

Our results showed that density treatments with and without physical contact result in remarkable changes in the microbiota composition at

the phylum, family, and genus levels. Higher housing densities without physical contact increase the relative abundances of Firmicutes, TM7, Prevotellaceae, Enterobacteriaceae, *Streptococcus*, *Prevotella*, *Escherichia*, and *Dorea*. More microbes from Firmicutes but fewer microbes from Bacteroidetes have been observed in patients with IBS than in healthy controls (Vila et al., 2018). Highly abundant microbes from TM7 are closely associated with host inflammatory mucosal diseases (Brinig et al., 2003). Microbes from Prevotellaceae are associated with chronic periodontitis (Kumar et al., 2003), and are more abundant in inflammatory bowel disease (IBD) (Kleessen et al., 2002; Lucke et al., 2006). Microbes from TM7 and Prevotellaceae are closely associated with human diseases (Elinav et al., 2011). Microbes from *Bacteroides* and *Prevotella* are negatively correlated with leptin levels in male rats (Queipo-Ortuno et al., 2013). Microbes from *Streptococcus* are closely associated with IBS (Vila et al., 2018), and microbes from *Dorea* are associated with diarrhea-predominant IBS (Maharshak et al., 2018). Microbes from *Escherichia* could invade the gut mucosal epithelium, resulting in bloody diarrhea and ulceration of the colon (Anderson et al., 2016). Higher housing density without physical contact also increases the relative abundances of *Faecalibacterium*, *Oscillospira*, and *Treponema*. Microbes from *Faecalibacterium* and *Streptococcus* have been associated with IBS symptoms (Vila et al., 2018), while those from *Bacteroides* and *Oscillospira* are found to be more abundant in non-obese diabetic mice than that in the control group (Ferreira et al., 2012). Microbes from *Oscillospira* are reported to play an important role in mediating destruction of the intestinal epithelial barrier, and then leading to the translocation of gut bacteria in obesity (Lam et al., 2012). Some microbes from *Treponema* have been associated with mammalian diseases (McKenna et al., 2008; Stanton and Canale-Parola, 1979). Moreover, crowding stress decreases the relative abundances of Bacteroidetes, *Halomonas* and *Alteromonas*. These bacteria are important in maintaining gut health (Lan et al., 2016; Lin et al., 2018; Mizuno et al., 2013; Vila et al., 2018).

High density treatment with physical contact increases the relative abundances of Ruminococcaceae, Erysipelotrichaceae, *Ruminococcus*, and *Bacteroides*. Similarly, microbes from these families or genera are closely associated with human diseases. Erysipelotrichaceae is considered to be one of the key families for aggravating intestinal inflammation and colonic disease (Palm et al., 2014; Zackular et al., 2013). Microbes from *Bacteroides* are generally symbiotic bacteria, but may also become opportunistic pathogens (Kumar and Sivaraman,

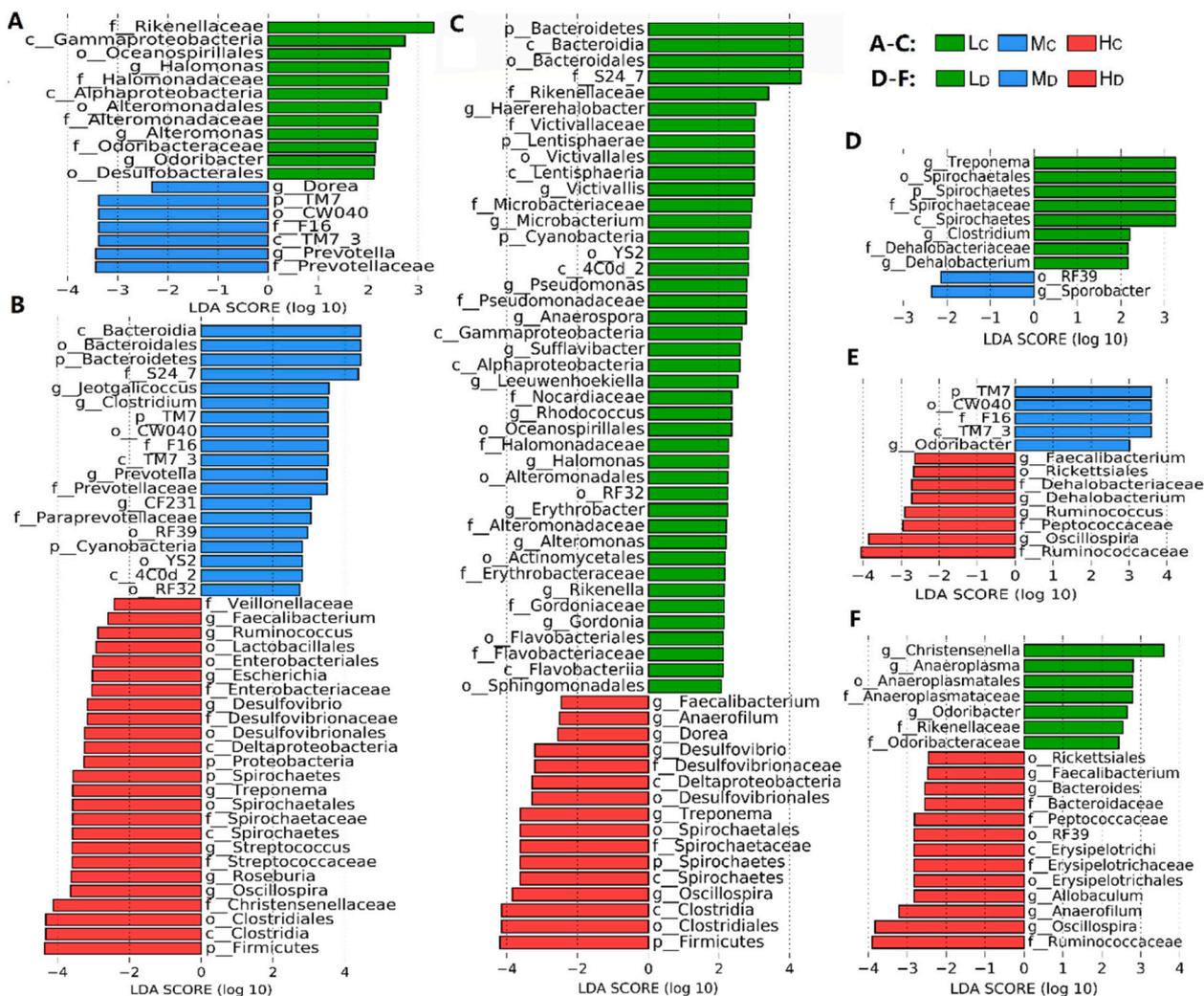


Fig. 3. Microbial taxa with significant differences between three treatment groups in laboratory density treatments without (A–C) and with (D–F) physical contact as revealed by LEfSe analysis with LDA score > 2 ($P < .05$). L_c, low-crowding group without physical contact (2 cages/group); M_c, medium-crowding group without physical contact (4 cages/group); H_c, high-crowding group without physical contact (25 cages/group); L_d, low-density group with physical contact (2 voles/cage); M_d, medium-density group with physical contact (4 voles/cage); H_d, high-density group with physical contact (8 voles/cage).

2011). The relative abundance of microbes from *Bacteroides* is higher in patients with IBD (Vila et al., 2018). Microbes from *Ruminococcus* are found in cancer patients (Chen et al., 2012), and the relative abundance of microbes from this genus is higher in impaired memory mice (Pyndt Jorgensen et al., 2014). High density treatment also increases the relative abundances of microbes from *Faecalibacterium* and *Oscillospira*. High density treatment decreases the relative abundances of microbes from *Christensenella* and *Odoribacter*. Abundance of *Christensenella* is lower in patients who develop subsequent bacteremia or bloodstream infection (Montassier et al., 2016). Microbes from *Christensenella* are significantly depleted in ulcerative colitis patients (Rajilic-Stojanovic et al., 2013) *Odoribacter* is responsible for the production of sulfonolipids, which potentially promote maturation of the intestine or immune system (Walker et al., 2017). This genus has been found to be enriched in the healthy group compared to in the metabolic syndrome group (Lim et al., 2017). In addition, our results also showed that housing densities alter the relative abundance of other taxa (specific details in Tables 1 and 2) in certain groups. In our study, we mainly focus on the common response to gut microbiota in two experiments. Brandt's voles are typically social animals and strict herbivores, which could result in different responses of gut microbial composition under density treatments. Future research should take into account the aspect and focus on the association between these microbes and diseases in voles.

It is notable that density treatments without physical contact show a stronger effect on gut microbes (as well as their association with hormones, see below) than do the density treatments with physical contact (Tables 1 and 2). This is unexpected because aggression due to physical contact and space restrictions exacerbates density-induced stress. It is likely that density experiments without physical contact impose extra stress because of social isolation, as compared to the density experiment with physical contact, which would reduce this stress. Indeed, a previous study indicated that social isolation could increase social stress (Pan et al., 2014). However, the stress hormone levels in density treatment without physical contact are lower than the density treatment with physical contact, indicating that a lack of physical contact reduced the stress level (see below). This contradicting observation of microbes and hormones requires further investigation in future studies.

The variance within groups (low and high density groups) in density treatments with physical contact is smaller than that within groups without physical contact (Fig. 2). However, the variance in the medium group is similar. This observation indicates that physical contact would increase the exchange of microbes between individuals in density experiments with physical contact, as compared to the density experiments without physical contact.

In general, our results indicated that high density significantly increases the relative abundances of the disease-associated microbes,

Table 1

The phyla and families of gut microbes in Brandt's voles showing significant variations in the relative abundances between density treatment groups ($P < .05$). "Yes" indicates significant difference; H_C, M_C, and L_C denote the high-, medium-, and low-crowding groups without physical contact, respectively. H_D, M_D, and L_D denote the high-, medium-, and low-density groups with physical contact, respectively. ">" indicates that the relative abundance is significantly larger.

Phylum/family	Hc	Hc	Mc	Lc	Mc	Lc	H _D	H _D	M _D	L _D	M _D	L _D
	>	>	>	>	>	>	>	>	>	>	>	>
	Lc	Mc	Lc	Hc	Hc	Mc	L _D	M _D	L _D	H _D	H _D	M _D
TM7			Yes		Yes						Yes	
Bacteroidetes				Yes	Yes							
Firmicutes	Yes	Yes										
Spirochaetes	Yes	Yes										Yes
Proteobacteria		Yes										
Lentisphaerae				Yes								
Cyanobacteria				Yes	Yes							
Alteromonadaceae				Yes		Yes						
Rikenellaceae				Yes		Yes				Yes		
Christensenellaceae		Yes										
Desulfovibrionaceae	Yes	Yes										
Halomonadaceae				Yes		Yes						
Odoribacteraceae						Yes				Yes		
Peptococcaceae							Yes	Yes				
Prevotellaceae			Yes		Yes							
Ruminococcaceae							Yes	Yes				
Spirochaetaceae	Yes	Yes										Yes
Streptococcaceae		Yes										
Victivallaceae				Yes								
F16			Yes		Yes						Yes	
S24_7				Yes	Yes							
Dehalobacteriaceae								Yes				Yes
Enterobacteriaceae		Yes										
Erysipelotrichaceae							Yes					
Microbacteriaceae				Yes								
Anaeroplasmataceae										Yes		
Bacteroidaceae							Yes					
Pseudomonadaceae				Yes								
Nocaydiaceae				Yes								
Erythrobacteraceae				Yes								
Gordoniaceae				Yes								
Flavobacteriaceae				Yes								
Veillonellaceae		Yes										
Paraprevotellaceae					Yes							

suggesting that high-crowding/density stress may reduce the fitness of animals under natural conditions.

4.2. Impact on stress hormones and immunity

Previous studies have indicated that social stress could change the dopamine and 5-HT concentrations (Palanza et al., 2001). Chronic mild stress increases the levels of plasma CORT in adult male Sprague Dawley rats (Garate et al., 2011). CORT and T are produced by the HPA

and HPG (hypothalamic-pituitary-gonadal) axes respectively, and are essential in mediating the aggressive behavior of males (Montoya et al., 2012). Aggression is important for individuals to maintain dominance (Briffa and Elwood, 2004; Careau et al., 2010; Haller et al., 2005; Seebacher et al., 2013). In addition, cortisol and testosterone could participate in social aggression and may jointly regulate social behavior, and serotonin may differentiate impulsive and instrumental aggression (Montoya et al., 2012). In this study, we found that Brandt's voles in high-density-induced stress with and without physical contact

Table 2

Genera of gut microbes in Brandt's voles showing significant variations in relative abundances between density treatment groups ($P < .05$), and the significant correlations between levels of serum hormones/immunity and the relative abundance of microbes at the genus level. "Yes" indicates significant difference. *, **, and *** indicate that the correlation coefficients are significant (* $P < .05$, ** $P < .01$, and *** $P < .001$). The coefficients within parenthesis () are from the density experiment with physical contact, the other coefficients are from the density experiment without physical contact. H_C, M_C, and L_C denote the high-, medium-, and low-crowding groups without physical contact, respectively. H_D, M_D, and L_D denote the high-, medium-, and low-density groups with physical contact, respectively. ">" indicates that the relative abundance is significantly larger.

Genus	H _C	H _C	M _C	L _C	M _C	L _C	H _D	H _D	M _D	L _D	M _D	L _D	CORT	5-HT	T	IgG
	>	>	>	>	>	>	>	>	>	>	>	>				
	L _C	M _C	L _C	H _C	H _C	M _C	L _D	M _D	L _D	H _D	H _D	M _D				
<i>Faecalibacterium</i>	Yes	Yes					Yes	Yes						0.43*		0.67***
<i>Oscillospira</i>	Yes	Yes					Yes	Yes					0.47*	0.50*(0.58*)		0.51*
<i>Treponema</i>	Yes	Yes										Yes	0.52**	0.46*	(-0.51*)	
<i>Dorea</i>	Yes		Yes													0.52**
<i>Odoribacter</i>						Yes				Yes	Yes					
<i>Ruminococcus</i>		Yes						Yes								
<i>Desulfovibrio</i>	Yes	Yes												0.43*		0.52**
<i>Anaerofilum</i>	Yes						Yes									
<i>Dehalobacterium</i>								Yes				Yes				
<i>Clostridium</i>					Yes							Yes				
<i>Halomonas</i>				Yes		Yes										
<i>Ateromonas</i>				Yes		Yes										
<i>Prevotella</i>			Yes		Yes											
<i>Bacteroides</i>							Yes							(0.50*)		
<i>Microbacterium</i>				Yes									-0.48*			
<i>Jeotgaliococcus</i>					Yes											
<i>Erythrobacter</i>				Yes												
<i>Sufflavibacter</i>				Yes												
<i>Pseudomonas</i>				Yes												
<i>Sporobacter</i>									Yes							(0.42*)
<i>Anaeroplasma</i>											Yes					
<i>Christensenella</i>											Yes					
<i>Allobaculum</i>							Yes									
<i>Escherichia</i>		Yes														
<i>Streptococcus</i>		Yes														0.41*
<i>Roseburia</i>		Yes														
<i>Haererehalobacter</i>				Yes												
<i>Victiallis</i>				Yes												-0.41*
<i>Anaerospira</i>				Yes												
<i>Leeuwenhoekiella</i>				Yes												
<i>Rhodococcus</i>				Yes												
<i>Rikenella</i>				Yes												
<i>Gordonia</i>				Yes												
<i>CF231</i>					Yes											

showed higher serum CORT and 5-HT concentrations, which is consistent with previous observations. Higher serum T concentrations are observed in the high density group with physical contact. Previous studies suggest that higher T concentrations may facilitate aggression in male rodents (Montoya et al., 2012).

Previous research has indicated that 5-HT in the bacterial production of neurotransmitter is related to *Streptococcus*, *Escherichia* and *Enterococcus* (Roshchina, 2010). Some intestinal microbes of mammals (e.g., *E. coli* and *Saccharomyces spp.*, *Candida*, *Streptococcus*, *Enterococcus spp.* and *Bacillus spp.*) are able to synthesize and release neurotransmitters (e.g., 5-HT and dopamine) and neuromodulators that could promote

enteroendocrine cells to produce neuropeptides (Lyte, 2013, 2014). *E. coli* is capable of producing tryptophan, and monoassociation with enteropathogenic *E. coli* could promote stress response (Sudo et al., 2004). *E. coli* infection can activate the HPA axis to produce circulating corticosterone by prostaglandin E2 from the immune system (Dinan and Cryan, 2012; Zimomra et al., 2011). CORT has been shown to be related to *L. helveticus* and *B. longum* (Messaoudi et al., 2011). In our study, density treatments without physical contact increase the relative abundances of *Escherichia* and *Streptococcus*. The relative abundances of microbes from *Streptococcus* and *Escherichia*, as well as the 5-HT levels are higher in the high density group without physical contact, which is consistent with the

previous observations (Roshchina, 2010). These results suggest that high density treatment increases stress by likely increasing specific microbes that produce 5-HT. In addition, we found that the levels of CORT and 5-HT are significantly correlated with the relative abundance of microbes from *Oscillospira* and *Treponema* in the density experiment without physical contact; The level of 5-HT is significantly correlated with the relative abundance of microbes from *Oscillospira* and *Bacteroides* in the density experiment with physical contact. The causal relationship between these changing microbes affected by different housing densities and CORT/5-HT requires further investigation.

The density-dependent prophylaxis hypothesis predicts that the immunity of animals should be elevated at high densities to counter the increasing risks of parasitic infection and pathogen transmission (Wilson and Reeson, 1998). Significantly higher serum IgG levels are observed under high and medium housing densities in female Brandt's voles (Li et al., 2003). Serum IgG could reduce the risk of bacterial translocation, intestinal damage, and systemic infection by entering the intestinal lumen and recognizing gram-negative bacteria (Zeng et al., 2016). Previous studies have indicated that social disruption elevates the concentration of IL-6, which is significantly negatively correlated with the relative abundance of *Coprococcus*, *Pseudobutyribrio*, and *Dorea* (Bailey et al., 2011). In our study, we found that high density groups both with and without physical contact significantly increase serum IgG concentrations, supporting the density-dependent prophylaxis hypothesis (Wilson and Reeson, 1998). The increased immunity is likely used to counter the increased relative abundance of the disease-associated microbes as observed in our study. Indeed, in the density experiment without physical contact, we found that the level of IgG is significantly positively related to the relative abundance of microbes from the following genera: *Faecalibacterium*, *Oscillospira*, *Dorea*, *Desulfovibrio*, and *Streptococcus*.

It is notable that the observed relationship between microbiota and hormones should be carefully interpreted because the interaction of microbiota and hormones should be bidirectional (Neuman et al., 2015). In our study, some causal relationships are observed. For example, high density without physical contact increases the relative abundances of specific microbes (e.g., from *Escherichia* and *Streptococcus*) that produce 5-HT and increases the 5-HT level. High density without physical contact increases the relative abundances of microbes from some genera (e.g., *Faecalibacterium*, *Oscillospira*, *Dorea*, *Desulfovibrio*, and *Streptococcus*) that are significantly and positively related to the IgG level. It is likely that the increase in CORT could increase the relative abundance of microbes from *Oscillospira* and *Treponema* in the high density groups without physical contact because cortisol could change the intestinal permeability and then the gut microbiota (Cryan and Dinan, 2012). Most observed associations between microbiota and hormone levels in this study require investigation by manipulative experiments to identify their causal relationships.

We found that stress hormone levels in the density experiment without physical contact are lower than those in the density experiment with physical contact (Fig. 1), indicating that a lack of physical contact reduced the stress level. This observation contradicts the observation that the changes in microbiota and their relationship with hormones are stronger in the density group without physical contact than in those with physical contact (Tables 1 and 2). Thus, the isolation effects of density experiments without physical contact are difficult to confirm from the currently available studies. We will examine this question in future studies.

4.3. Implications for population regulation and management

To the best of our knowledge, the density-dependent roles of gut microbiota in the population regulation of small rodents have not been well investigated. In this study, we found that a high population density could alter the gut microbiota of Brandt's voles by likely increasing the relative abundance of disease-associated microbes which may contribute to population decline when the population reaches to peak density. 5-HT, a neurotransmitter or neurohormone, is critical for

modulating host behavior (Spohn and Mawe, 2017). We found that high density increases the 5-HT levels which would increase the aggressive behavior of animals and thus contribute to population regulation. Some high-density-induced gut microbes may have potential to be involved in the control population of rodents when their numbers are affecting humans. Our results may be useful in studying the gut microbiota-induced psychological disorders or diseases of livestock or humans living in highly crowded conditions, and finding solutions for reducing stress-induced diseases by regulating their gut microbiota.

5. Conclusions

In this study, our results provide new insights into the density-induced host-gut microbiota interactions. We found that high density grouping can elevate the levels of hormones (CORT and 5-HT), IgG, and/or T, and induce significant variations in colon microbiota compositions related to stress-related disorders, which in turn affect the host's neuroendocrine and immune systems. Remarkable variation in microbiota compositions consistently demonstrates increased community diversity in disease-associated microbes in high density groups. In addition, many density-induced microbes are found to be associated with stress hormones and immunity. Future studies should examine the causal relationship between the changes in microbes and levels of hormones and immunity, as well as their effects on individual fitness and population regulation.

Author contributions

Z. Z. designed the research. J. L. conducted the density experiment; J. L. and S. H. conducted the density experiment without physical contact; J.L. and S. H. collected the data of the density experiment without physical contact; J.L., S. H., J. Z, and W. L collected the data of the density experiment with physical contact. G. L provided the support for data analysis. J. L., Z. Z. and G. L wrote the manuscript. All authors contributed to manuscript writing and gave final approval for publication.

Data availability statement

The raw sequence data are uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with accession number SRR9208392.

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Declaration of competing interest

There were no conflicts of interests for this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2020.104838>.

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