

Title page

Title: Exposure to soil, house dust, and decaying plants increases gut microbial diversity and decreases serum IgE levels in BALB/c mice

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Running title: Impact of living-environment sanitation on gut flora

Summary

To assess the impact of sanitation of a living environment on gut microbiota and development of the immune system, we raised BALB/c mice under three distinct environmental conditions: a specific-pathogen-free animal room (SPF), general animal room (XZ), and farmhouse (JD). All other variables like diet, age, genetic background, physiological status, and original gut microbiota were controlled for in the three groups. Using high-throughput sequencing of the 16S rRNA gene, we found that each mouse group had a specific structure of the gut microbial community. Groups JD and XZ harbored a significantly more diverse and richer gut microbiota than did group SPF. Bacteroidetes were significantly more abundant in groups XZ and JD than in group SPF, whereas Firmicutes showed the inverse pattern. Total serum IgE levels were significantly lower in groups XZ and JD than in group SPF. There were no significant differences in gut microbiota diversity and serum IgE concentration between groups JD and XZ, but we found higher abundance of dominant genera in the gut microflora of group JD. We conclude that exposure to soil, house dust, and decaying plant material enhances gut microbial diversity and innate immunity. Our results seem to provide new evidence supporting the hygiene hypothesis.

Introduction

The gut microbiome is an essential “organ” of the body (O’Hara *et al.*, 2006), assisting the host with a variety of functions ranging from digestion and protection against pathogens to host immunity and regulation of the central nervous system. Although the majority of published studies have focused on the effect of nutrition on the human gut microbiota (Bäckhed *et al.*, 2005; De Filippo *et al.*, 2010), other factors can also influence the gut microbiome, e.g., genetic background, the mode of delivery, medical treatments, and geographical provenance (Lagier *et al.*, 2012). Nonetheless, the effects of exposure to unsanitary living environment on gut microbiota diversity and composition remain largely unclear (Adlerberth *et al.*, 2007; Penders *et al.*, 2006).

According to the “hygiene hypothesis,” higher standards of personal cleanliness have reduced the rates of early-life infection, thereby impeding natural development of the immune system and predisposing children to allergic diseases (Strachan, 1989). Similarly, the modified “microflora hypothesis” states that the overly hygienic Western lifestyle limits exposure to microbes and alters colonization of the infant’s gut, disrupting the development of the immune system and ultimately leading to allergic diseases (Wold, 1998). In support of this hypothesis, many studies have shown that 1) early-life exposure to an unsanitary living environment protects against the development of allergic diseases (Ownby *et al.*, 2002; Hesselmar *et al.*, 1999; Litonjua *et al.*, 2002; Ball *et al.*, 2000), 2) reduced diversity of gut microbiota during infancy is associated with allergic diseases later in childhood (Abrahamsson *et al.*, 2012; Vebo *et al.*, 2011; Bisgaard *et al.*, 2011; Vael *et al.*, 2011), and 3) appropriate intestinal microbial stimuli during early life are crucial for launching an immunoregulatory network that protects from induction of IgE at mucosal sites (Cahenzli *et al.*, 2013). On the basis of the modified microflora hypothesis,

we surmised that less exposure to environmental microorganisms negatively influences the gut microbiota and is the main cause of allergic diseases.

The aim of this study was to determine the impact of exposure to an unsanitary living environment on the diversity and composition of mouse gut microbiota and on development of the immune system. We used BALB/c mice as study subjects because this is an inbred strain and the individuals have an identical genetic makeup. Variables like diet, age, physiological status, and original gut microbiota were controlled for in experimental mice, except for these variables: bedding material and sanitary living conditions. Allergen sensitization was assessed by measuring total serum IgE levels after administration of 2-4-dinitrofluorobenzene (DNFB) (Kim *et al.*, 2011).

Results

1. *Exposure to soil, house dust, and decaying plants increased gut microbial richness and diversity*

The maintenance of sufficient bacterial richness and diversity is important for providing the gut microbiota with functional redundancy, adaptability, and systematic robustness in the face of environmental challenges. With a total of 1 132 715 eligible sequences, we rarified each sample down to 29 645 reads (97% threshold) in order to estimate bacterial richness and diversity (Supporting Information Table S1). The rarefaction curve showed that mice in the “farmhouse” (JD) and “general animal room” (XZ) groups had a significantly higher number of the detected bacterial species than did mice in the “specific-pathogen-free animal room” (SPF; $P < 0.01$; Fig. 1A and B, Table S2) group. As an alternative method for estimation of richness, we employed the nonparametric Chao1 estimator in 97% of operational-taxonomic-unit (OTU) clustering. In

line with the rarefaction data from the identified OTUs, groups XZ and JD had a significantly higher number of estimated OTUs than did group SPF ($P < 0.01$; Fig. 1C, Table S2); this result indicated that groups XZ and JD had higher bacterial richness than did the SPF mice. There was no significant difference between groups JD and XZ. To estimate microbial diversity, we used two ecological indices: Shannon and phylogenetic diversity (PD). Shannon measurements showed that groups XZ and JD had significantly higher bacterial diversity than did group SPF ($P < 0.05$; Fig. 1D, Table S2). Similarly, the PD index revealed that groups XZ and JD harbored a more diverse gut microbiota than the SPF mice did ($P < 0.01$; Fig. 1E, Table S2). Both indices showed no significant differences in microbial diversity between groups JD and XZ. Altogether, these findings suggested that exposure to unsanitary living environment was linked to increased richness and diversity of the gut microflora.

2. *Exposure to soil, house dust, and decaying plants inhibited DNFB-induced elevation of the serum IgE level*

The hygiene hypothesis suggests that exposure to unsanitary environment protects from allergic diseases, which are characterized by elevated total serum IgE levels (William, 2004; Kim *et al.*, 2009). To test whether exposure to an unsanitary environment suppresses elevation of total serum IgE levels, we treated our murine groups with DNFB. After the fifth DNFB treatment on day 13, serum samples were collected and total IgE levels were measured using an enzyme-linked immunosorbent assay (ELISA). At the same time, the weights of the body, spleen, and liver were recorded. In all the DNFB-treated mice, total serum IgE levels were significantly higher than those in untreated (negative control) mice ($P < 0.05$; Fig. 2, Table S3). Compared to the findings for group SPF, a significantly lower total serum IgE level was observed in groups

XZ and JD ($P < 0.01$; Fig. 2A, Table S3), but there was no significant difference between the latter two (Table S3). We also assessed the spleen/body and liver/body weight ratios of the three DNFB-treated groups and of the untreated mice (there were no significant differences in body weight among the mice kept under different living conditions). The results showed that the spleen/body weight ratio in groups SPF, XZ, and JD was higher than that in negative control mice ($P < 0.05$; Table S3). The spleen/body weight ratio of group JD was significantly lower than that of group SPF ($P < 0.05$; Fig. 2B, Table S3), while there was no significant difference between groups XZ and JD (Table S3). The liver/body weight ratio was significantly higher in group SPF than in groups JD and XZ ($P < 0.01$; Fig. 2C, Table S3). There was no statistically significant difference in the liver/body weight ratio between groups JD and XZ (Table S3). The liver/body weight ratio of the JD mice was not significantly different from that of negative control mice (Table S3), whereas the liver/body weight ratio in group XZ was higher ($P < 0.05$; Table S3).

To determine whether gut microbial richness/diversity correlates with changes in total serum IgE levels, the spleen/body weight ratio, liver/body weight ratio, gender, or the living conditions, we performed body weight matched-pair correlation analysis. Our results indicated that the Chao1 estimator, Shannon diversity index, and PD significantly correlated with total serum IgE levels, the spleen/body weight ratio, and liver/body weight ratio (Table 2). The gender did not correlate with any of the richness/diversity indices (Table 2). These results suggested that exposure to unsanitary living environment enhanced immune function possibly by changing the diversity and richness of the gut microbial community.

3. *Exposure to soil, house dust and decaying plants changed microbial communities at the phylum level*

For detailed taxonomic analysis, individual sequences were classified by means of the RDP classifier (Cole *et al.*, 2009), which assigned approximately 97% of all sequences to 31 known phyla. Firmicutes, Bacteroidetes, and Actinobacteria were dominant, and Proteobacteria and Tenericutes were also relatively abundant (Table S4). These five phyla comprised an average of 99% of all classifiable sequences across the samples. Comparison of mean abundance levels among the groups by means of the two-tailed least significant difference (LSD) test showed that the abundance of each phylum varied substantially among groups JD, XZ, and SPF. The phylum Firmicutes was significantly more abundant in group SPF than in groups XZ and JD ($P < 0.05$; Fig. 3A, Table S4), and there was no significant difference between groups XZ and JD (Table S4). In contrast, phylum Bacteroidetes was significantly more abundant in group XZ than in group JD and significantly more abundant in group JD than in group SPF ($P < 0.05$; Fig. 3B, Table S4). Furthermore, the phylum Actinobacteria was significantly more abundant in group JD than in group XZ ($P < 0.05$; Fig. 3C, Table S4); however, there was no significant difference between groups JD and SPF (Table S4). The phylum Tenericutes was significantly more abundant in group JD than in groups SPF and XZ ($P < 0.05$; Table S4), and no significant difference was observed between groups XZ and SPF (Table S4).

In addition, we performed canonical correlation analysis (CCoA) to evaluate the possible association of the top 18 most abundant phyla with total serum IgE, the liver/body weight ratio, and spleen/body weight ratio. Top 18 most abundant phyla were selected due to the limitations of the canonical correlation analysis (CCoA) algorithm and software (SAS 9.1). When the number of phyla exceeded 18, the output results reported an error. The results showed that the 18 most abundant phyla were significantly associated with total serum IgE and the liver/body weight ratio ($P < 0.05$; Fig. 4, Table S5A). Standardized canonical coefficients indicated that the phyla

Bacteroidetes and Firmicutes had the strongest effects on the first axis, followed by Actinobacteria, Proteobacteria, and “unclassified” (Fig. 4, Table S5B). This finding indicates that these five phyla correlated most strongly with the total serum IgE level. Although the adjusted canonical correlation coefficient between the top 18 phyla and the three immune function indices (total serum IgE, the liver/body weight ratio, and spleen/body weight ratio) was 0.992355, this result was not statistically significant. The main reason that the result was not significant with such a high correlation coefficient is that the data on 16S rDNA high-throughput sequencing had higher dispersion due to inter-individual variability.

4. *Exposure to soil, house dust and decaying plants changed microbial communities at the genus level*

Among the 526 genera identified using the RDP classifier, the genera *Lactobacillus*, *Staphylococcus*, *Bacteroides*, *Enterorhabdus*, *S24-7_norank*, *Brachybacterium*, *Parabacteroides*, *Desulfovibrio*, *Acinetobacter*, and the *RC9_gut_group* were the top 10 most abundant genera in the three mouse groups (Table S6). The genus *Lactobacillus* was present at very high levels in two SPF samples, representing up to 52% of all sequences, and the prevalence in these two samples contributed to low diversity (Fig. 5).

We compared the mean abundance of each top genus among the groups by the two-tailed LSD test. The results showed that the genera *Lactobacillus*, *Enterorhabdus*, and *Acinetobacter* were significantly more abundant in group SPF than in groups XZ and JD ($P < 0.05$; Fig. S2, Fig. S3, Table S6). In contrast, the *S24-7_norank* and *RC9_gut_group* were significantly less abundant in group SPF than in groups XZ and JD ($P < 0.05$; Fig. S2, Fig. S3, Table S6). The genus *Bacteroides* was significantly less abundant in mice from groups SPF and JD than in the

XZ mice ($P < 0.01$; Fig. S2, Fig. S3, Table S6). Conversely, the genera *Staphylococcus*, *Brachybacterium*, and *Desulfovibrio* were significantly less abundant in groups SPF and XZ than in group JD ($P < 0.01$; Fig. S2, Fig. S3, Table S6). The abundance of the genus *Parabacteroides* was not significantly different among the three groups (Fig. S3, Table S6).

Among the 526 identified genera, there were 60 genera whose abundance levels were significantly different among the different living conditions ($P < 0.05$; Fig. S3). The differences in the gut microbiome at the genus level between the three groups are shown in Fig. S3.

Taken together, these results showed that fewer number of genera were present in the cecal contents of the SPF mice. In contrast, more number of genera were found in the JD mice, which had been exposed to the most unsanitary living environment.

5. Gut microbial community profile was associated with exposure to soil, house dust, and decaying plant material

In an effort to identify systematic differences in microbial communities among groups SPF, XZ, and JD, the top 100 genera were hierarchically clustered based on their relative abundance across the samples (Fig. S4). Although there seemed to be a difference in the relative abundance of the most dominant genera between groups JD and XZ (Fig. S2), the majority of clusters showed a similar pattern between these two groups and a difference between JD/XZ and the SPF mice (Fig. S4). The first cluster of five genera that included *Yaniella* showed, in general, greater abundance in groups JD and XZ than in group SPF (Fig. S4). In addition, these genera shared a similar pattern within each group, indicating a coherent relationship among these genera. Another cluster that was enriched in *Anaerofustis* (Fig. S4) displayed a pattern negatively correlating with the *Yaniella* cluster within groups JD and XZ (correlation coefficient $r = 0.9936$,

$P < 0.01$). There were other clusters with differing patterns between JD/XZ and SPF, such as the *Dietzia*, *Arthrobacter*, and *Microbacterium* clusters. These results suggested that the community-wide interrelationship of the gut microbiota was altered in the SPF mice.

Next, we assessed differences in the total bacterial community at the single-sample level by clustering the SPF, JD, and XZ samples according to their bacterial genera as identified by the RDP classifier (Fig. S5). Hierarchical cluster analysis showed that the bacterial communities were subdivided into three subclusters and that groups JD and XZ were at a close distance. We also used sequence information from the 16S ribosomal RNA gene to calculate pairwise UniFrac distances between the microbial communities. We assessed both relative abundance data (weighted analysis) and the presence/absence status (unweighted analysis). Unsupervised clustering by means of principal coordinates analysis (PCoA) of UniFrac distance matrices indicated that environmental exposure primarily explained the variation in our dataset, where the SPF, JD, and XZ microbiota got separated along the principal coordinate (Fig. 6). In summary, these results demonstrated that the profile of the gut microbial community was affected by environmental exposure.

Discussion

In this study, we characterized the impact of exposure to soil, house dust, and decaying plants (campus and farm) on the diversity and composition of the gut microbiota and immune function of BALB/c mice, using high-throughput 16S rDNA sequencing and DNFB sensitization. We found that housing sanitation conditions have distinct effects on the richness and diversity of the gut microbiota as well as on the development of the immune system. Our results indicate that

immune homeostasis is linked to the diversity and richness of the gut microbiota, which, in turn, were affected by housing sanitation, as stated in the hygiene and microflora hypotheses.

In Western countries, the human living environment, nutrition, and lifestyle have changed immensely during the last few decades. For example, diets now contain more fat and protein, and people spend ~92% of their time indoors (Ott, 1989). Consequently, the burden of chronic inflammatory diseases, including atopic diseases, has increased dramatically. In a study by De Filippo *et al.* (2010), the composition of the gut microflora in rural African children was compared with that of European children. These authors found higher diversity of the gut microbiota in rural African children than in European children, and composition of the gut microbiome was also significantly different. More recently, Alexander *et al.* (2013) reported that human gut microbial communities in Russian rural regions are dominated by *Ruminococcus bromii*, *Bifidobacterium adolescentis*, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, and the genera *Coprococcus* and *Roseburia*, which are associated with intestinal health. In mice, higher diversity of gut bacteria protects from autoimmune disorders (Ochoa *et al.*, 2010). Our results suggest that exposure to an unsanitary living environment also plays an important role in shaping the gut microbiota, thus reducing the occurrence of chronic inflammatory diseases.

Our results indicate that sanitation is as important for shaping the gut microbiota as diet is. We assumed that the host's living conditions, to which the microbes are exposed, and the host's diet are the main source of the gut microbiota. Frequent changes in nutrition and in the gut environment, together with the use of antibiotics, often lead to the disappearance of some species from the gut microflora. Therefore, it is necessary to replenish the intestines with bacterial culture strains in order to maintain gut microbial stability, which may be compromised in oversanitized living environments. On the other hand, similar to the effects of nutrients in culture

media used for in vitro analysis, nutrition determines which new bacterial culture strains would survive in the intestines.

Our results also indicate that sanitation influences mammalian immunity. We theorize that exposure to microbes from the living environment or bedding is similar to inoculation of the animal skin, mucosa, or body lumens with microbes, and this approach is expected to improve immunity and increase immune tolerance of gut microbes, with subsequent high diversity of gut microbes. At the same time, the high diversity of gut microbes should also improve host immunity.

In this study, we analyzed the diversity and composition of the bacterial community using high-throughput sequencing technology and found changes in the gut bacterial community that are caused by housing sanitation. Other microorganisms, such as microbial eukaryotes, helminths, and viruses in the bedding of our mice may have a similarly strong influence on the host immunity and structure of the gut microbial community. In addition, large or small organic/inorganic molecules in the bedding or dust may affect host gut immunity and influence the gut microbiota.

Discrepancies among results of 16S rDNA sequence analysis are common and can be caused by several factors, such as differences in analytical methods (e.g., high-throughput 16S rDNA sequencing versus phylogenetic microarray analysis) and in sample sources, uneven coverage of target sequences of microbes by different PCR primers, and differences in the number of sequence reads. The analytical method may be a particularly important factor. For example, using sequence analysis of 16S rRNA gene libraries, Mulder *et al.* (2009) found that the Firmicutes-to-Bacteroidetes ratio in pigs housed in a natural outdoor environment was higher than that in pigs housed in a hygienic indoor environment. In contrast, using high-throughput

sequencing of 16S rDNA in fecal samples, De Filippo *et al.* (2010) found the opposite trend in rural African children versus European children, with African children showing a higher Bacteroidetes-to-Firmicutes ratio. Using the same method, we also found that groups of mice exposed to microbe-rich living environments (JD and XZ) had a higher Bacteroidetes-to-Firmicutes ratio than SPF mice.

Among the genera identified in our study, *Lactobacillus* was significantly more abundant in the gut microbiota of group SPF than of groups JD and XZ (25%, 11%, and 2%, respectively). The prevalence of *Lactobacillus* in SPF samples most likely contributes to the low diversity and abundance of other genera that are important immune modulators. For example, abundance of the genus *Bacteroides* was 0.87%, 2.36%, and 10.5% in the gut microbiota of the SPF, JD, and XZ mice, respectively. The microbiota interacts with the host immune system, causing accumulation of several lymphocyte subsets at mucosal sites. Recent reports showed that induction of each lymphocyte subset can be regulated by a distinct component of the microbiota (Atarashi *et al.*, 2011). For instance, *Clostridium* clusters IV and XIVa promote accumulation of T_{reg} cells in the colon (Atarashi *et al.*, 2011), whereas segmented filamentous bacteria (SFBs) strongly induce intestinal T helper 17 (T_H17) cells, which participate in host resistance to intestinal pathogens and promote systemic autoimmunity (Gaboriau *et al.*, 2009; Wu *et al.*, 2010). It is well known that various *Lactobacilli* are effective probiotics that efficiently modulate the development of the immune system (Mohamadzadeh *et al.*, 2005). They cannot replace other beneficial intestinal microbes that perform similarly important functions in the regulation of the immune system. For instance, Atarashi *et al.* (2011) found that accumulation of T_{reg} cells in the small intestinal lamina propria is differentially regulated by commensal microorganisms and that the induction of T_{reg} cells depends on microorganisms with specialized properties. This

observation may explain why the total serum IgE concentration (used as an indicator of immune function) is higher in the SPF mice than in groups XZ and JD, although the probiotic genus *Lactobacillus* is more abundant in the gut microbiota of the SPF mice.

Many studies have shown inverse associations between early-life exposure to pet animals and allergic diseases (Ownby *et al.*, 2002; Hesselmar *et al.*, 1999). According to a questionnaire survey of more than 2000 Swedish children, exposure to pets during the first year of life is associated with lower prevalence of allergic rhinitis and asthma at school age (Hesselmar *et al.*, 1999). Here, we found higher abundance of dominant genera in the gut microflora of the JD mice than in that of groups XZ and SPF (Fig. S3). Nonetheless, there were no significant differences in microbiota diversity and the total serum IgE concentration between groups JD and XZ. This result suggests that exposure to farm animals changed the gut microbiota composition in the JD mice but not the diversity and richness. Therefore, the diversity and richness of gut microbiota may be affected by sanitation conditions rather than the source of the microbes. Similarly, Azad (2013) did not find a significant difference in microbiota richness and diversity between infants with pets and infants without pets.

In this research, we used the SPF mouse model although the SPF conditions are not completely equivalent to the modern human lifestyle. The SPF mice were fed autoclaved food and sterilized water and lived in a sterilized cage with autoclaved bedding, under dust-free living conditions. Nevertheless, they were in contact with their feces and urine, which are rich in many kinds of microbes. Modern humans are regularly exposed to dust and soil and consume food and water that have not been autoclaved but transmission of microbes via the fecal-oral route is largely prevented. Therefore, the SPF mice are not a perfect experimental model but still a good one.

In conclusion, we analyzed the gut microbiota of three groups of mice exposed to different levels of environmental sanitation. We found that the three groups show significantly different structures of the gut microbial community. By comparing group SPF with groups JD and XZ, we found that high levels of environmental sanitation reduce the diversity of the gut microbiota and worsen host immune function. Moreover, by comparing groups JD and XZ, we found that exposure to farm animals changes the composition of the gut microbiota but does not affect its richness and diversity.

Experimental Procedures

1. Animals and groups

Six-week-old male and female BALB/c mice were purchased from B & K Universal Ltd. (Shanghai, China; Fig. S1). The mice were randomly distributed into three groups. Mice in the control group (SPF) were raised in cages with sterile-grade murine bedding in a specific pathogen-free animal room in Nanjing City. The second group of mice (XZ) was raised in a general animal room of Nanjing Xiaozhuang University in Nanjing City. The cage bedding was composed of decaying hay, decaying fallen leaves, and 25 g of top soil from the campus (Table 1). In addition, 25 g of room dust was collected and evenly placed in the cages. The last group of mice (JD) was reared in a farmhouse of a rural area in Jiang Du County, which is ~100 km away from Nanjing City. This small farm had two dogs, six goats, 20 hens, and several ducks. The cage bedding was composed of decaying hay, decaying wheat straw, decaying fallen leaves, and 25 g of top soil from the farm (Table 1). The house dust also was put into the cages. The dirty bedding was removed every 3 weeks.

All the experiments involving animals were performed in an air-conditioned animal room ($24^{\circ}\text{C} \pm 2^{\circ}\text{C}$; standard 12-hour light/12-hour dark cycle; relative humidity, $40\% \pm 5\%$); the mice were fed the same type of SPF diet and distilled water. They were allowed free access to tap water and food in the form of a standard pellet diet. The mice were bred in a male:female ratio of 1:3, and two females were shifted to a separate cage when they were found to be pregnant. The F1 progeny were weaned at 18–21 days of age and were shifted to separate cages. The females and males were caged separately. Ten animals were kept in one cage before five animals were euthanized to collect cecum samples at 28–31 days of age.

All experiments involving mice were approved by the National Institute of Biological Sciences and by the Animal Care Research Advisory Committee of Southeast University; the experiments were conducted in accordance with the guidelines of the Animal Research Ethics Board of Southeast University (Approval Number, 2013021505).

When the parent mice (P0) were 7 weeks old, we started the experiment by breeding at a male:female ratio of 1:3 (Fig. S1). There were 36 pups (17 males and 19 females) from 6 dams in the JD group, 33 pups (17 males and 16 females) from 6 dams in the XZ group, and 44 pups (20 males and 24 females) from 9 dams in the SPF group. Two animals in a matched pair had the same gender, similar body weight, and were born of the same dam. After these conditions were met, 10 pairs of animals (five pairs of males and five pairs of females) were randomly selected from the F1 offspring of each group's dams for the experiment. The negative control group of animals (five males and five females) was selected randomly from the F1 offspring of the SPF group's dams.

We euthanized the mice and collected cecum samples when the offspring (F1) reached 28–31 days of age (Fig. S1) and then analyzed the diversity of the gut microbial community based on sequencing of the 16s rDNA gene.

2. Allergen sensitization and challenge

Allergic dermatitis was induced in the first filial generation (F1) of mice aged 6–7 weeks by repeated topical application of DNFB as described previously (Tomimori *et al.*, 2002) (Figure S1). Briefly, 25 µl of 0.15% DNFB in acetone/olive oil (3:1) was applied to the inner and outer surfaces of the ears, and 100 µl of the same solution was applied to the shaved back skin once on days 1 and 4. On days 7, 10, and 13, the sensitized mice were challenged by applying 0.2% DNFB to the back and ear skin surfaces. The negative control group was treated with the same volumes of the solvent, acetone/olive oil (3:1).

The mice were euthanized 24 h after the final DNFB application, and the weights of the body, liver, and spleen were recorded.

3. Serum IgE measurements

On day 14, 24 h after the final DNFB application, we prepared total serum samples. Total serum IgE concentration was measured using the eBioscience Mouse IgE Ready-SET-GO mouse ELISA kit (Affymetrix Co.). In summary, immunoplate (Corning Costar 9018 96-well plate) microwells were coated with 100 µl per well of an anti-mouse IgE monoclonal antibody diluted 250-fold with coating buffer. The plates were sealed and incubated overnight at 4°C. After liquid was aspirated from the wells and after three washes with 400 µl of PBS containing 0.05% Tween 20 (wash buffer), the plates were blocked with 250 µl of blocking buffer for 2 h at room

temperature. Subsequently, the wells were washed three times with washing buffer, and 100 μ l of each standard or serum samples (diluted 100- or 200-fold with Assay Buffer A) was added to the appropriate wells. After incubation for 2 h at room temperature, the seals were removed and the wells were washed four times with washing buffer. Next, 100 μ l of a freshly prepared solution of a streptavidin-horseradish peroxidase-conjugated detection antibody was added to each well, and the plates were incubated for 1 h at room temperature. After four washes with washing buffer, 100 μ l of a substrate solution (0.1 M sodium citrate, tetramethylbenzidine dihydrochloride, and H_2O_2) was added. The plates were incubated for 15 min at room temperature in the dark. The enzymatic reaction was terminated by adding 100 μ l of a stop solution (1 M H_2SO_4) to each well, and absorbance was immediately measured at 450 nm on an ELISA reader (EL 800; Bio-Tek).

4. DNA extraction

The gut contents ($n = 8$ per treatment group) were removed from the cecum and frozen in PBS 24 h before storage at -80°C until DNA extraction. The genomic DNA extraction procedure was based on a protocol proposed by Zoetendal *et al.* (2006). After resuspension of the content of each cecal sample in 1 ml of the physiological solution (0.9% w/v sodium chloride) and after homogenization by vigorous manual shaking, 300 μ l of the suspension was centrifuged ($10\,000 \times g$ for 10 min at 4°C) to obtain pellets. The pellets were dissolved in 5 ml of ice-cold $1\times$ PBS and centrifuged at $700 \times g$ at 4°C for 1 min. The supernatants were transferred into a 15-ml tube and were centrifuged at $9000 \times g$ at 4°C for 5 min. Subsequently, the pellets were resuspended in 300 μ l of $10\times$ TE buffer (10 mM Tris-HCl PH=8.0, 1 mM EDTA PH=8.0) by repeated pipetting. Then, 100 μ l of lysozyme (200 mg/ml) was added, with incubation for 1 h at 37°C . Subsequently, 50 μ l of 10% SDS (wt/vol) and 20 μ l of proteinase K (20 mg/ml) were added. The

samples were incubated for 2 h at 50°C, after which 100 µl of NaCl (5M) was added, and the samples were incubated at 65°C for 10 min. An equal volume of phenol/chloroform (50:50) was added, and the samples were shaken well until the phases were completely mixed. The mixtures were centrifuged at $4500 \times g$ for 2 min. The upper layer was then transferred into a new tube. This step was repeated again so that the interface of the two layers was clean. Next, 1/10 volume of 3 M sodium acetate, pH 5.2, and two volumes of 95% ethanol were added, with gentle mixing. The mixtures were centrifuged at 4°C and $9000 \times g$ for 20 min. The precipitated genomic DNA was washed twice in 1 ml of 70% ethanol. The dried samples were resuspended in 50–100 µl of H₂O. DNA quality was assessed by gel electrophoresis and spectrophotometry (OD 260/280). Extracted DNA was stored at –20°C.

5. MiSeq sequencing analysis of the community structure of gut microflora

We sent extracted genomic DNA to Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). For each sample, the 16S rRNA gene was amplified using the following primer set: 515F (5'-GTGCCAGCMGCCGCGG) and 907R (5'-CCGTCAATTCMTTTRAGTTT), as described by Angenent *et al.* (2005). These PCR primers target the V4 hypervariable 16S rRNA region. The amplification was performed as follows. A typical 50-µl amplification reaction included 30 µl of distilled H₂O, 10 µl of 5× TransStart FastPfu buffer, 5 µl of a dNTP mix (2.5 mM of each dNTP), 1 µl of each primer (10 µM forward and 10 µM reverse primers), 1 µl of TransStart FastPfu Taq DNA Polymerase (TransGen Biotech), and 2 µl (300 ng) of template DNA. The PCR conditions were as follows: 94°C for 3 min for DNA denaturation, with the amplification lasting 30 cycles (94°C for 45 s, 50°C for 45 s, and 72°C for 45 s), and final extension for 5 min at 72°C. Each sample was amplified in

duplicate, and the resulting duplicate samples were combined, gel purified using the AxyPrepDNA Gel Purification Kit (AXYGEN, Tewksbury MA, USA), and then quantified using the QuantiFluor™-ST Handheld Fluorometer with UV/Blue Channels (Promega Corporation). Sequencing was conducted on Illumina MiSeq at Shanghai Majorbio Bio-pharm Technology Co. Ltd., generating 250-bp paired-end reads. The paired sequences were combined using the FLASH software (<http://ccb.jhu.edu/software/FLASH>) set to the minimum overlap of 10 bp. Other parameters were at default settings. Using Trimmomatic, we denoised PCR artifacts by eliminating low-quality sequences (Bolger *et al.*, 2014). Using UPARSE (Edgar, 2013), the quality-checked sequences were clustered into *de novo* OTUs at the 97% similarity threshold for production of OTUs. The most abundant sequence in each OTU was chosen as a representative sequence. We removed chimeric sequences using UCHIME (Edgar *et al.*, 2011). We used usearch_global to map the reads to the OUTs and obtained an OTU abundance table for each sample. We removed one outlier with a very low sequence count and microbial diversity.

Then, we rarefied all the other samples by randomly sampling 29 645 sequences from each sample 10 times in order to match the sequence numbers across the samples. Either normalized sequence counts from individual subsamples or the average counts of eight subsamples were used for further analyses, as indicated in the description of each analysis. We used the Mothur software to construct rarefaction curves and calculate ecological indices of the Chao1 estimator and Shannon diversity/richness (Schloss *et al.*, 2009). We measured the PD index using the Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso *et al.*, 2010). We classified sequences by means of the RDP Classifier software at the 70% confidence threshold for sequences longer than 200 bp (Wang *et al.*, 2007). We performed clustering on genera obtained from the RDP Classifier by means of the complete linkage hierarchical

clustering technique using the R package hclust (<http://sekhon.berkeley.edu/stats/html/hclust.html>). To examine dissimilarities in community composition, we performed PCoA in QIIME. PCoA, where a distance matrix is used to plot n samples in $(n - 1)$ -dimensional space, was used to compare groups of samples based on unweighted and weighted UniFrac distance metrics.

6. Statistical analyses

All calculations (e.g., analysis of variance [ANOVA] and linear correlation analysis) were performed with the SPSS software, version 18.0. Canonical correlation analysis (CCoA) was conducted using the SAS 9.1 software.

Acknowledgments

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References

Abrahamsson, T.R., Jakobsson, H.E., Andersson, A.F., Bjorksten, B., Engstrand, L., and Jenmalm, M.C. (2012) Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol* 129:434–440.

- Adlerberth, I., Strachan, D.P., Matricardi, P.M., Ahrne, S., Orfei, L., Aberg, N., *et al.* (2007) Gut microbiota and development of atopic eczema in 3 European birth cohorts. *J Allergy Clin Immunol* 120:343–350.
- Alexander, V.T., Elena, S.K., Anna, S.P., Maxim, S.B, Alexander, V.P, Andrey, K.L., *et al.* (2013) Human gut microbiota community structures in urban and rural populations in Russia. *Nat Commun* 4: 2469-2477
- Angenent, L.T., Kelley, S.T., Amand, A.S., Pace, N.R. and Hernandez, M.T. (2005) Molecular identification of potential pathogens in water and air of a hospital therapy pool. *Proc Natl Acad Sci* 102: 4860-4865.
- Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y. *et al.* (2011) Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 331:337-41.
- Azad, M.B., Konya, T., Maughan, H., Guttman, D.S., Field, C.J., Sears, M.R. *et al.* (2013) Infant gut microbiota and the hygiene hypothesis of allergic disease: impact of household pets and siblings on microbiota composition and diversity. *Allergy Asthma Clin Immunol* 9:15-23.
- Bäckhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., and Gordon, J.I. (2005) Host-bacterial mutualism in the human intestine. *Science* 307:1915-20.
- Ball, T.M., Castro-Rodriguez, J.A., Griffith, K.A., Holberg, C.J., Martinez, F.D., and Wright, A.L. (2000) Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. *N Engl J Med* 343:538–543.
- Bisgaard, H., Li, N., Bonnelykke, K., Chawes, B.L., Skov, T., Paludan-Muller, G., *et al.* (2011) Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol* 128:646–652.

Bolger, A.M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114-20.

Cahenzli, J., Köller Y., Wyss M., Geuking M.B., McCoy K.D. (2013) Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host Microbe* 14:559-70.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data., *Nat Methods* 7: 335–336.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., *et al.* (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* 37:D141-5.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., *et al.* (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci USA* 107:14691-6.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27:2194-200.

Edgar, R.C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 10:996-8.

Gaboriau-Routhiau, V., Rakotobe, S., Lécuyer, E., Mulder, I., Lan, A., Bridonneau, C., *et al.* (2009) The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* 31:677-89.

Hesselmar, B., Aberg, N., Aberg, B., Eriksson, B., and Björkstén, B. (1999) Does early exposure to cat or dog protect against later allergy development? *Clin Exp Allergy* 29:611-7.

Hesselmar, B., Aberg, N., Aberg, B., Eriksson, B., and Bjorksten, B. (1999) Does early exposure to cat or dog protect against later allergy development? *Clin Exp Allergy* 29:611–617.

Kalliomaki, M., Kirjavainen, P., Eerola, E., Kero, P., Salminen, S., and Isolauri, E. (2001) Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* 107:129–134.

Kim, D.Y., Jung, J.A., Kim, T.H., Seo, S.W., Jung, S.K., and Park, C.S. (2009) Oral administration of *Uncariae rhynchophylla* inhibits the development of DNFB-induced atopic dermatitis-like skin lesions via IFN-gamma down-regulation in NC/Nga mice. *J Ethnopharmacol* 122:567-72.

Kim, G.D., Kim, T.H., Jang, A.H., Ahn, H.J., Park, Y.S., and Park, C.S. (2011) α -Lipoic acid suppresses the development of DNFB-induced atopic dermatitis-like symptoms in NC/Nga mice. *Exp Dermatol* 20:97-101.

Lagier, J.C., Million, M., Hugon, P., Armougom, F. and Raoult, D. (2012) Human gut microbiota: repertoire and variations. *Front Cell Infect Microbiol* 2:136-54.

Litonjua, A.A., Milton, D.K., Celedon, J.C., Ryan, L., Weiss, S.T., and Gold, D.R. (2002) A longitudinal analysis of wheezing in young children: the independent effects of early life exposure to house dust endotoxin, allergens, and pets. *J Allergy Clin Immunol* 110:736–742.

Mohamadzadeh, M., Olson, S., Kalina, W.V., Ruthel, G., Demmin, G.L., Warfield, K.L., *et al.* (2005) Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proc Natl Acad Sci USA* 102:2880-5.

Mulder, I.E., Schmidt, B., Stokes, C.R., Lewis, M., Bailey, M., Aminov, R.I., *et al.* (2009) Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. *BMC Biol* 7:79-98.

- Nakayama, J., Kobayashi, T., Tanaka, S., Korenori, Y., Tateyama, A., Sakamoto, N., *et al.* (2011) Aberrant structures of fecal bacterial community in allergic infants profiled by 16S rRNA gene pyrosequencing. *FEMS Immunol Med Microbiol* 63:397–406.
- O'Hara, A.M. and Shanahan, F. (2006) The gut flora as a forgotten organ. *EMBO Rep* 7:688–693.
- Ochoa-Repáraz, J., Mielcarz, D.W., Wang, Y., Begum-Haque, S., Dasgupta, S., Kasper, D.L., *et al.* (2010) A polysaccharide from the human commensal *Bacteroides fragilis* protects against CNS demyelinating disease. *Mucosal Immunol* 3:487–95.
- Ott, W.R.(1989) Human activity patterns: A review of the literature for estimating time spend indoors, outdoors and in transit. *Proceedings of the Research Planning Conference on Human Activity Patterns*, EPA/600/4-89/004(EPA National Exposure Research Laboratory, Las Vegas), 3.
- Ownby, D.R., Johnson, C.C., and Peterson, E.L. (2002) Exposure to dogs and cats in the first year of life and risk of allergic sensitization at 6 to 7 years of age. *JAMA* 288:963–972.
- Penders, J., Thijs, C., van den Brandt, P.A., Kummeling, I., Snijders, B., Stelma, F., *et al.* (2007) Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. *Gut* 56:661–667.
- Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, I., *et al.* (2006) Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* 118:511–521.
- Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B. *et al.* (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities., *Appl Environ Microbiol* 75: 7537–7541.

Strachan, D.P. (1989) Hay fever, hygiene, and household size. *BMJ* 299:1259–1260.

Tomimori, Y., Muto, T., Fukami, H., Saito, K., Horikawa, C., Tsuruoka, N., *et al.* (2002) Chymase participates in chronic dermatitis by inducing eosinophil infiltration. *Lab Invest* 82:789–794,

Vael, C., Vanheirstraeten, L., Desager, K.N., and Goossens, H. (2011) Denaturing gradient gel electrophoresis of neonatal intestinal microbiota in relation to the development of asthma. *BMC Microbiol* 11:68-75.

Vebo, H.C., Sekelja, M., Nestestog, R., Storro, O., Johnsen, R., Oien, T., *et al.* (2011) Temporal development of the infant gut microbiota in IgE sensitized and non-sensitized children determined by the GA-map infant array. *Clin Vaccine Immunol* 18:1326–1335.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microb* 73: 5261-5267.

William, C. (2004) The immunogenetics of asthma and eczema: a new focus on the epithelium. *Nature Reviews Immunology* 4:978-988

Wold, A.E. (1998) The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? *Allergy* 53:20–25.

Wu, H.J., Ivanov, I.I., Darce, J., Hattori, K., Shima, T., Umesaki, Y., *et al.* (2010) Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* 32:815-

Zoetendal, E.G., Booiijink, C.C., Klaassens, E.S., Heilig, H.G., Kleerebezem, M., Smidt, H., *et al.* (2006) Isolation of RNA from bacterial samples of the human gastrointestinal tract. *Nat Protoc* 1:954–959.

Table 1. Living environment and diet for each group of mice.

Group/ treatment	SPF	XZ (Xiao Zhuang)	JD (Jiang Du)
Diet	SPF diets		
Water	Sterilized tap water		
Cage density	≤ 5		
Room conditions	Specific pathogen- free animal room	General animal room	Farmhouse
Room temperature	24°C $\pm$ 2°C	24°C $\pm$ 2°C	24°C $\pm$ 2°C
Location	Nanjing City	Nanjing City	Jiangdu, 100 km from Nanjing City
Bedding	Sterilized bedding (pelletized cardboard; the main ingredient was birchwood)	25 g of house dust, decaying hay, decaying fallen leaves, 25 g of top soil collected from the University campus	25 g of house dust, decaying hay, decaying fallen leaves, decaying wheat straw, and 25 g of top soil collected from the farmyard where animals (dogs, hen, ducks, and goats) were present
P0 mice	7 weeks old (at breeding)		
F1 Test1	6 weeks old during DNFB treatment		

F1 Test2	Cecal microflora analysis was based on high-throughput sequencing of 16S rDNA
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Table 2. Correlation between microbial richness/diversity and some variables among all samples.

(A) Chao1 estimator

		ENVIRONMENT	SPLEEN_R	LIVER_R	IgE	Gender
Pearson	R value	.602 ^{**}	-.528 ^{**}	-.524 ^{**}	-.666 ^{**}	.111
	P value	.002	.008	.009	.000	.605
Spearman	R value	.511 [*]	-.541 ^{**}	-.398	-.712 ^{**}	.081
	P value	.011	.006	.054	.000	.707

(B) Shannon diversity index

		ENVIRONMENT	SPLEEN_R	LIVER_R	IgE	Gender
Pearson	R value	.629 ^{**}	-.407 [*]	-.691 ^{**}	-.530 ^{**}	.251
	P value	.001	.048	.000	.008	.236
Spearman	R value	.524 ^{**}	-.482 [*]	-.541 ^{**}	-.554 ^{**}	.093
	P value	.009	.017	.006	.005	.665

(C) Phylogenetic diversity (PD)

		ENVIRONMENT	SPLEEN_R	LIVER_R	IgE	Gender
Pearson	R value	.550**	-.536**	-.464*	-.631**	.075
	P value	.005	.007	.022	.001	.726
Spearman	R value	.524**	-.548**	-.395	-.724**	.044
	P value	.009	.006	.056	.000	.840

P values are from Student's two-tailed *t* test (**P* < 0.05, ***P* < 0.01; n = 8)

Fig. 1. Comparison of bacterial richness and diversity among control (SPF), Xiao Zhuang (XZ), and Jiang Du (JD) mice ($n = 8$; $*P < 0.05$, $**P < 0.01$ according to two-tailed least significant difference test). The SPF mice were raised in a specific-pathogen-free animal room, XZ mice were in a general animal room, and JD mice were in a farmhouse.

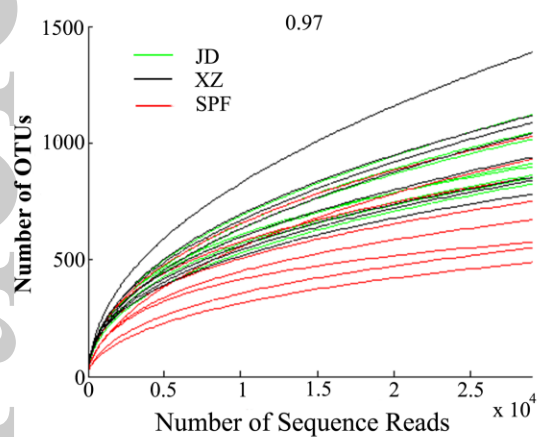
A. Rarefaction curves showing unique operational taxonomic units (OTUs) at the 97% threshold (a box graph at the rarefied sequence number).

B. Chao1 estimators.

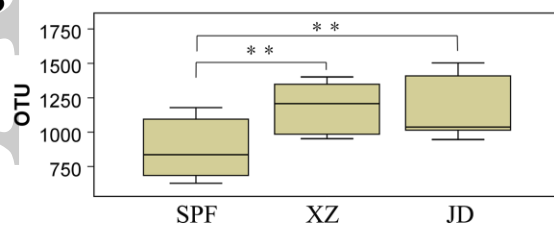
C. Shannon index.

D. Phylogenetic diversity (PD).

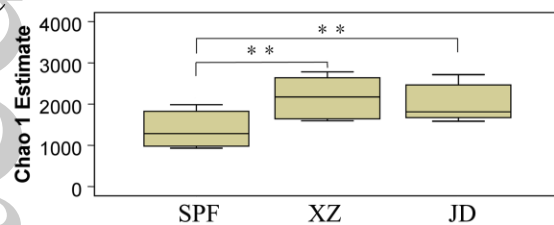
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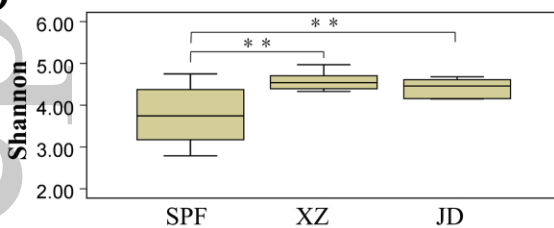
B



C



D



E

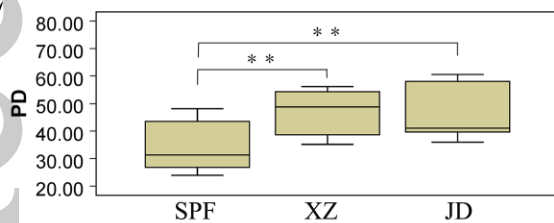


Fig. 2. Effects of exposure to different environmental conditions on (A) the total serum IgE level, (B) the spleen/body weight ratio, and (C) the liver/body weight ratio in BALB/c mice treated with 2-4-dinitrofluorobenzene (DNFB). Serum absorbance in each group was averaged and each bar represents mean $\pm$ SEM ($n = 10$; $*P < 0.05$, $**P < 0.01$ according to the two-tailed least significant difference test).

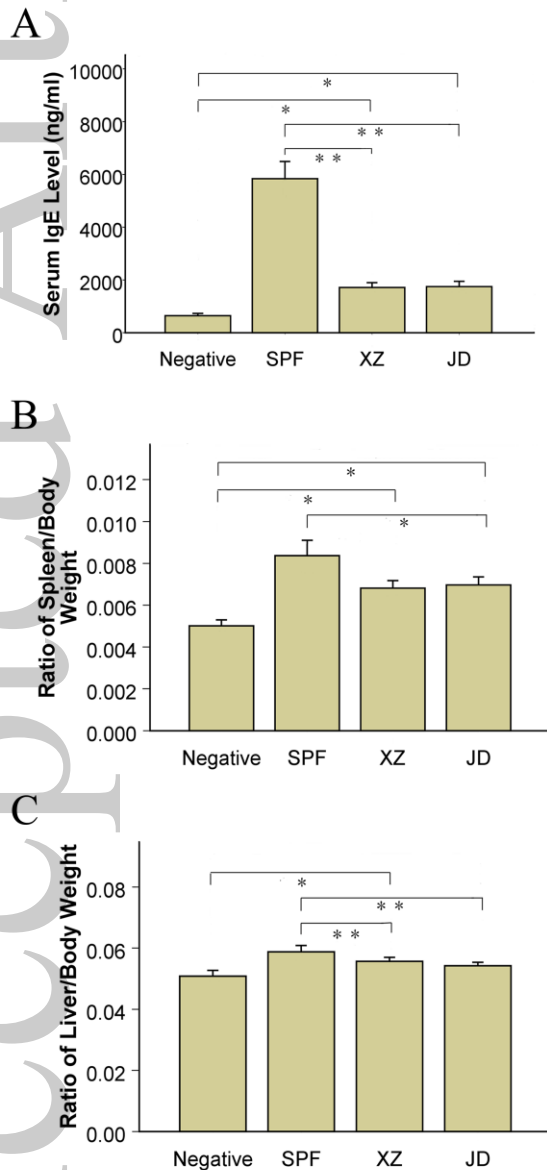


Fig. 3. Phylum-level comparison of the gut microbiota among the control (SPF), Jiang Du (JD), and Xiao Zhuang (XZ) groups. Percentages of (A) Firmicutes, (B) Bacteroidetes, and (C)

Actinobacteria (n = 8; * $P < 0.05$, ** $P < 0.01$ according to two-tailed least significant difference test).

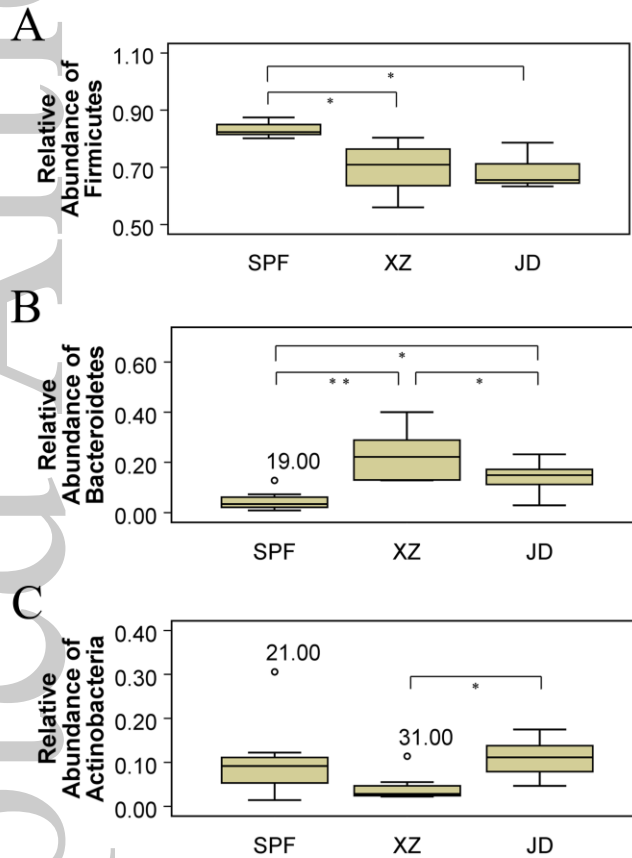


Fig. 4. Canonical correlation analysis (CCoA) between the top 18 most abundant phyla and immune function indices. The 1st axis is the linear combination of the abundance levels of top 18 most abundant phyla. The 2nd axis is the linear combination of the concentration of serum IgE, the ratios of spleen/body weight, and the ratios of liver/body weight. Colored spots represent the three groups of mice: SPF (control), Xiao Zhuang (XZ), and Jiang Du (JD).

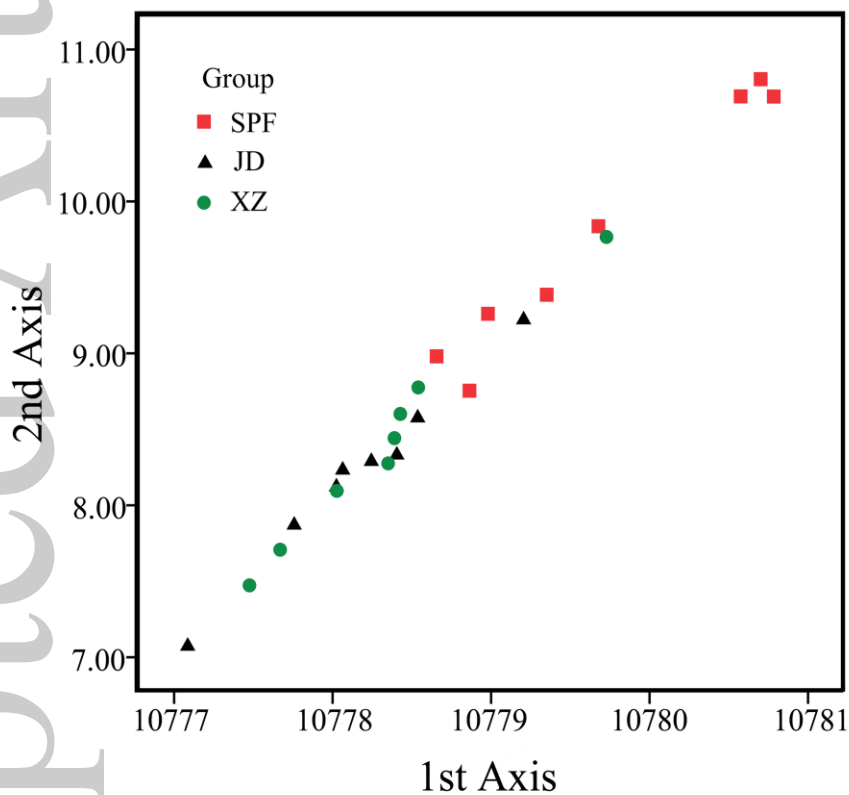


Fig. 5. Detailed relative abundance of bacterial genera in three most abundant major phyla of the gut microbiota.

A. Firmicutes

B. Bacteroidetes

C. Actinbacteria

Mouse groups: SPF (control), Jiang Du (JD) and Xiao Zhuang (XZ). Each bar represents an individual mouse.

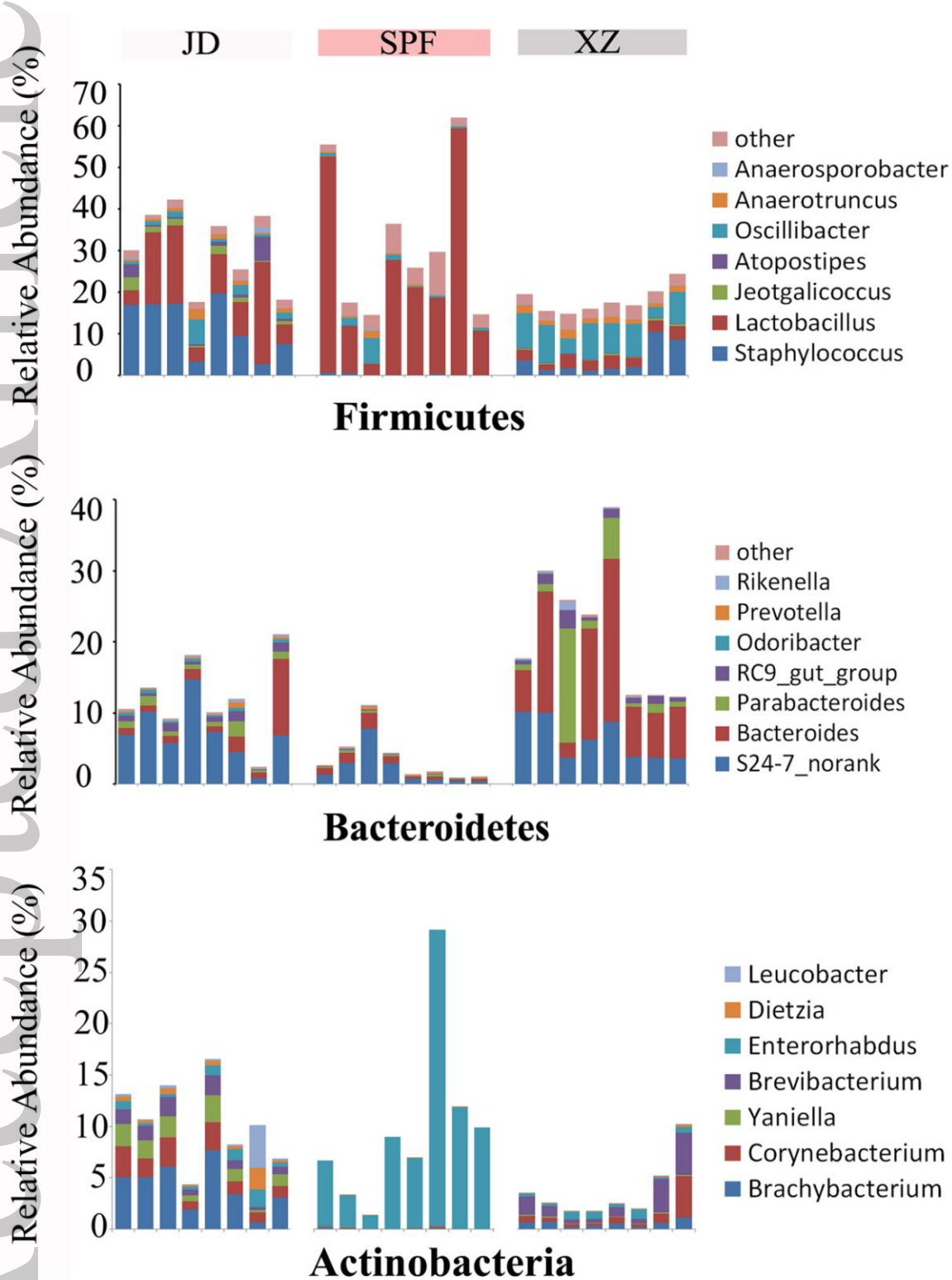
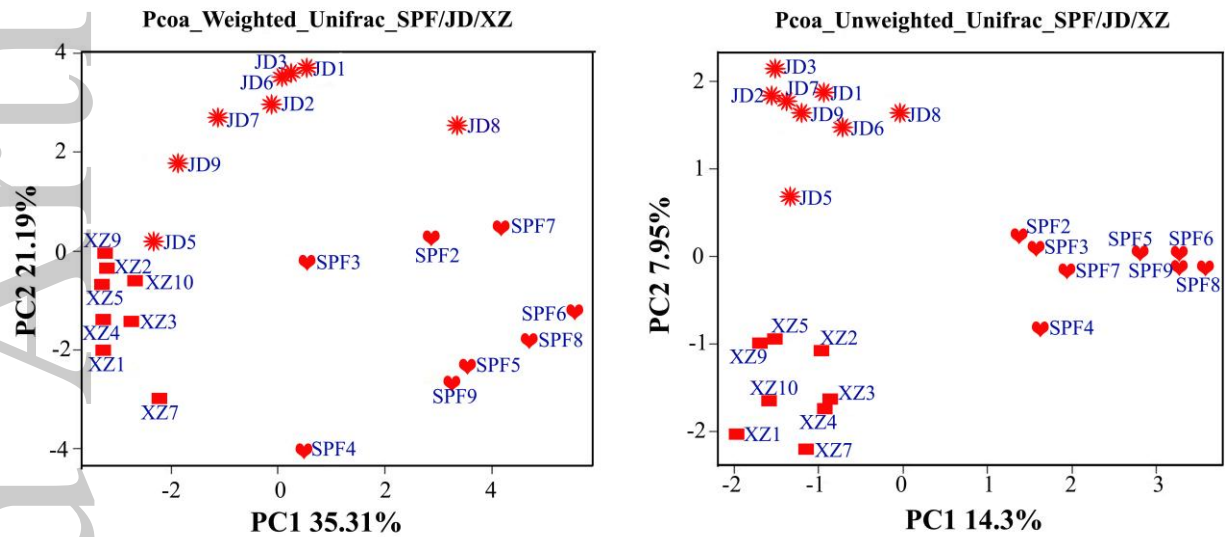


Fig. 6. Principal coordinates analysis (PCoA) of weighted and unweighted UniFrac distances for the cecal microbiota of the three mouse groups: SPF (control), Jiang Du (JD), and Xiao Zhuang (XZ). Analysis was based on the Illumina bacterial 16S rRNA gene dataset (V4 region).



1 **Supporting Information**

2

3 Table S1. Miseq data summary with the number of total sequence reads and qualified
4 sequence reads. Mouse groups: SPF (control), Xiao Zhuang (XZ), and Jiang Du (JD).

SAMPLE	MOUSE GENDER	TOTAL SEQUENCES	QUALIFIED SEQUENCES
JD1	male	78518	62612
JD2	male	67460	50286
JD3	female	60806	44596
JD5	female	59135	40797
JD6	female	65005	48008
JD7	male	55906	40622
JD8	female	70971	59255
JD9	female	51281	39446
SPF2	female	64925	53578
SPF3	female	50138	38287
SPF4	female	73540	48355

SPF5	female	52920	40344
SPF6	female	74214	70584
SPF7	male	55205	50249
SPF8	male	62081	54793
SPF9	male	52096	43434
XZ1	male	68643	43546
XZ2	male	62079	45660
XZ3	female	73173	50245
XZ4	male	65236	42557
XZ5	female	73102	54554
XZ7	female	62308	29645
XZ9	female	55363	37958
XZ10	female	67883	43304

1

2

3

- 1 Table S2. Microbial diversity indices with OTUs obtained by UCLUST (97%
- 2 similarity).
- 3 (A) Chao1 estimator

(I)GROUP (J)GROUP	MEAN DIFFERENCE (I-J)	STD. ERROR	SIG.(P-V ALUES)	95% CONFIDENCE INTERVAL	
				Lower Bound	Upper Bound
SPF JD	-637.00000**	211.01716	0.005	-1068.5785	-205.4215
SPF XZ	-773.50000**	211.01716	0.001	-1205.0785	-341.9215
JD XZ	-136.50000	211.01716	0.523	-568.0785	295.0785

- 4
- 5 (B) Shannon diversity index

(I)GROUP (J)GROUP	MEAN DIFFERENC E (I-J)	STD. ERRO R	SIG. (P-VALUE S)	95% CONFIDENCE INTERVAL	
				Lower Bound	Upper Bound
SPF JD	-0.64500*	0.24143	0.012	-1.1388	-0.1512

SPF	XZ	-0.80625**	0.24143	0.002	-1.3	-0.3125
JD	XZ	0.16125	0.24143	0.509	-0.3325	0.6550

1

2 (C)Phylogenetic Diversity (PD)

(I)GROUP (J)GROUP	MEAN DIFFERENCE (I-J)	STD. ERROR	SIG. (P-VALUES)	95% CONFIDENCE	
				INTERVAL	
				Lower Bound	Upper Bound
SPF JD	-12.34019**	4.21433	0.007	-20.9595	-3.7209
SPF XZ	-12.42587**	4.21433	0.006	-21.0452	-3.8066
JD XZ	-0.08569	4.21433	.984	-8.7050	8.5336

3

4 (D)Number of OTUs

(I)GROUP (J)GROUP	MEAN DIFFERENCE (I-J)	STD. ERROR	SIG. (P-VALUES)	95% CONFIDENCE INTERVAL	
				Lower Bound	Upper Bound

SPF	JD	-291.62500**	94.18679	0.004	-484.2586	-98.9914
SPF	XZ	-299.62500**	94.18679	0.003	-492.2586	-106.9914
JD	XZ	-8.00000	94.18679	.933	-200.6336	184.6336

1

2 *P*-values are from two-tailed least significant difference (**P* < 0.05, ***P* < 0.01; n =

3 8).

4

1 Table S3. Comparison of (A) total serum IgE levels, (B) spleen/body weight ratio, and
 2 (C) liver/body weight ratio among groups SPF (control), Xiao Zhuang (XZ), and
 3 Jiang Du (JD).

4 (A)

(I)GROUP (J)GROUP	MEAN DIFFERENCE (I-J)	STD. ERROR	SIG. (P-VALUES)	95% CONFIDENCE INTERVAL	
				Lower Bound	Upper Bound
SPF JD	4173.53104**	508.91906	.000	3138.1274	5208.9347
SPF XZ	4121.81460**	493.72401	.000	3117.3255	5126.3037
JD XZ	-51.71643	508.91906	.920	-1087.1201	983.6872
Negative SPF	-5191.81665**	470.74737	.000	-6149.5594	-4234.0739
Negative JD	-1018.28561*	486.66036	.044	-2008.4036	-28.1677
Negative XZ	-1070.00205*	470.74737	.030	-2027.7448	-112.2593

5

6 (B)

(I)GROUP	MEAN	STD.	SIG.	95% CONFIDENCE
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(J)GROUP	DIFFERENCE (I-J)	ERROR	(P-VALUES)	INTERVAL	
				Lower Bound	Upper Bound
SPF JD	.001552589*	.000713754	.036	.00010359	.00300159
SPF XZ	.001399888	.000713754	.058	-.00004911	.00284889
JD XZ	.000152701	.000713754	.832	-.00129630	.00160170
Negative SPF	-.003353598**	.000733313	.000	-.00484230	-.00186489
Negative XZ	-.001801009*	.000733313	.019	-.00328971	-.00031231
Negative JD	-.001953711*	.000733313	.012	-.00344241	-.00046501

1

2 (C)

(I)GROUP	MEAN	STD.	SIG.	95% CONFIDENCE	
(J)GROUP	DIFFERENCE (I-J)	ERROR	(P-VALUES)	INTERVAL	
				Lower Bound	Upper Bound
SPF JD	.014896269**	.002159091	.000	.01051308	.01927946

SPF XZ	.013472289**	.002159091	.000	.00908910	.01785548
JD XZ	-.001423980	.002159091	.514	-.00580717	.00295921
Negative SPF	-.018305100**	.002218255	.000	-.02280840	-.01380180
Negative JD	-.003408831	.002218255	.133	-.00791213	.00109447
Negative XZ	-.004832812*	.002218255	.036	-.00933611	-.00032952

1

2 *P*-values are from two-tailed least significant difference (**P* < 0.05, ***P* < 0.01; n =

3 8).

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- 1 Table S4. Top 10 most abundant phyla in mice gut microbiome. Comparison among
- 2 groups SPF (control), Jiang Du (JD), and Xiao Zhuang (XZ).

PHYLUM	MEAN			P-VALUE		
	JD	SPF	XZ	SPF-JD	SPF-X Z	JD-XZ
Firmicutes	0.673	0.7943	0.6981	0.013	0.043	0.581
Bacteroidetes	0.1383	0.0448	0.2266	0.015	0	0.021
Actinobacteria	0.11373 6	0.10212	0.04179 7	0.703	0.057	0.026
Proteobacteria	0.0509	0.0451	0.0248	0.724	0.22	0.12
Tenericutes	0.0078	0.0037	0.0035	0.035	0.949	0.03
Unclassified	0.0071	0.0027	0.0027	0.338	0.988	0.346
Candidate_division_TM 7	0.0046	0.0008	0.0006	0.185	0.95	0.166
Cyanobacteria	0.0032	0.0056	0.0003	0.311	0.034	0.235
Acidobacteria	0.00036 9	0.00034 2	0.00063 5	0.932	0.359	0.403

Chloroflexi	0.0004	0.0001	0.0002	0.179	0.505	0.485
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2 *P*-values are from two-tailed least significant difference ($*P < 0.05$, $**P < 0.01$; n =

3 8).

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- 1 Table S5. Canonical correlation analysis (CCoA) between top most abundant 18 phyla
2 and immunologic function indices (A), and standardized canonical coefficients (B).

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4 (A) Canonical Correlation Analysis

	CORRELATION	ADJUSTED CORRELATION	P VALUE
Ratio of L/B, IgE	0.994077	0.989667	0.0296
Ratio of S/B, Ratio of L/B, IgE	0.995726	0.992355	0.1479

5

6 (B) Standardized Canonical Coefficients

	BACTERO IDETES	FIRMICUTE S	ACTINOBA CTERIA	PROTEOBACTER IA	UNCLASSI FIED	CANDIDATE_DI VISION_TM7
Ratio of S/B, Ratio of L/B, IgE	1066.593	1055.58	690.5152	343.8801	93.9243	58.8839
Ratio of L/B, IgE	1096.367	1084.965	709.8321	353.5037	96.5691	60.4948
	Cyanobacte ria	Tenericutes	Acidobacteri a	Chloroflexi	Verrucomicr obia	Nitrospirae

Ratio of S/B, Ratio of L/B, IgE	52.8362	42.508	8.6728	5.0841	3.6638	2.607
Ratio of L/B, IgE	54.302	43.5921	9.0372	5.4658	3.6008	2.7268
	Chlorobi	Armatimonad etes	Planctomyce tes	Candidate_division _OD1	WCHB1_60	Gemmatimonadetes
Ratio of S/B, Ratio of L/B, IgE	1.121	0.8645	0.157	-0.762	-1.5654	-2.0744
Ratio of L/B, IgE	1.1471	1.0144	-0.0199	-0.7023	-1.7493	-2.2827

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- 1 Table S6. Top 20 most abundant genera in mice gut microbiome. Comparison among
- 2 SPF (control), Jiang Du (JD), and Xiao Zhuang (XZ).

GENUS	MEAN			P-VALUE		
	SPF	JD	XZ	SPF-JD	SPF-XZ	JD-XZ
Lactobacillus	0.253574	0.112098	0.02661	0.035	0.002	0.188
Staphylococcus	0.001842	0.117077	0.037456	0	0.125	0.002
Bacteroides	0.008738	0.023586	0.105422	0.524	0	0.002
Enterorhabdus	0.096356	0.007201	0.004821	0.002	0.001	0.924
S24-7norank	0.021427	0.071178	0.062272	0.006	0.02	0.589
Brachybacterium	0.000494	0.04114	0.005579	0	0.454	0
Parabacteroides	0.001986	0.009635	0.034217	0.631	0.053	0.132
Desulfovibrio	0.007221	0.025897	0.002512	0.009	0.474	0.002
Acinetobacter	0.020475	0.000714	0.000966	0.045	0.047	0.979
RC9_gut_group	0.000836	0.007857	0.010896	0.009	0	0.224
Brevibacterium	0.000254	0.011435	0.015295	0.019	0.003	0.391
Oscillibacter	0.015029	0.017161	0.068412	0.839	0	0

Corynebacterium	0.000419	0.018237	0.009163	0.001	0.068	0.059
Atopostipes	0.000306	0.015451	0.001057	0.015	0.897	0.02
Anaerotruncus	0.004537	0.010238	0.014871	0.064	0.002	0.127
Roseburia	0.005626	0.005454	0.012909	0.951	0.015	0.013
Helicobacter	0.004838	0.011726	0.008382	0.321	0.606	0.627
Alistipes	0.006844	0.009874	0.003926	0.315	0.333	0.056
Coprococcus	0.002894	0.002112	0.005692	0.401	0.006	0.001
<u>Yaniella</u>	<u>0.000159</u>	<u>0.014627</u>	<u>0.000971</u>	0.000	<u>0.739</u>	<u>0.000</u>

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2 *P*-values are from two-tailed least significant difference (**P* < 0.05, ***P* < 0.01; n =

3 8).

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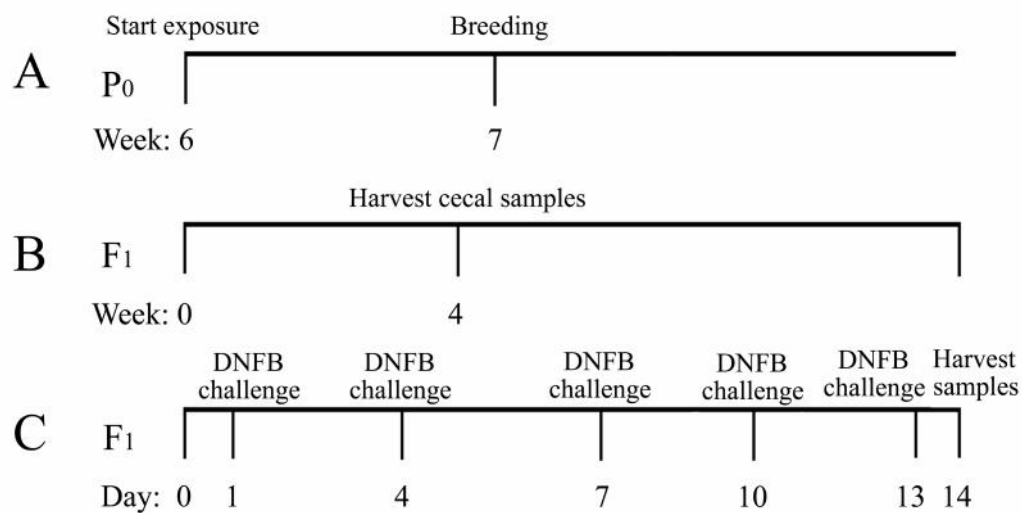
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1 Fig. S1 Experimental design.

2 A. Parental mice (6-week-old) were divided into three groups and exposed to
3 different environmental condition. One week later, mouse breeding was carried out.

4 B. First filial generation (F1) mice (4 to 5-week-old) were euthanized and cecum
5 samples collected.

6 C. F1 mice (6 to 7-week-old) were treated with 2-4-dinitrofluorobenzene (DNFB) on
7 days 1, 4, 7, 10, and 13. On day 14, blood, spleen, and liver were harvested.

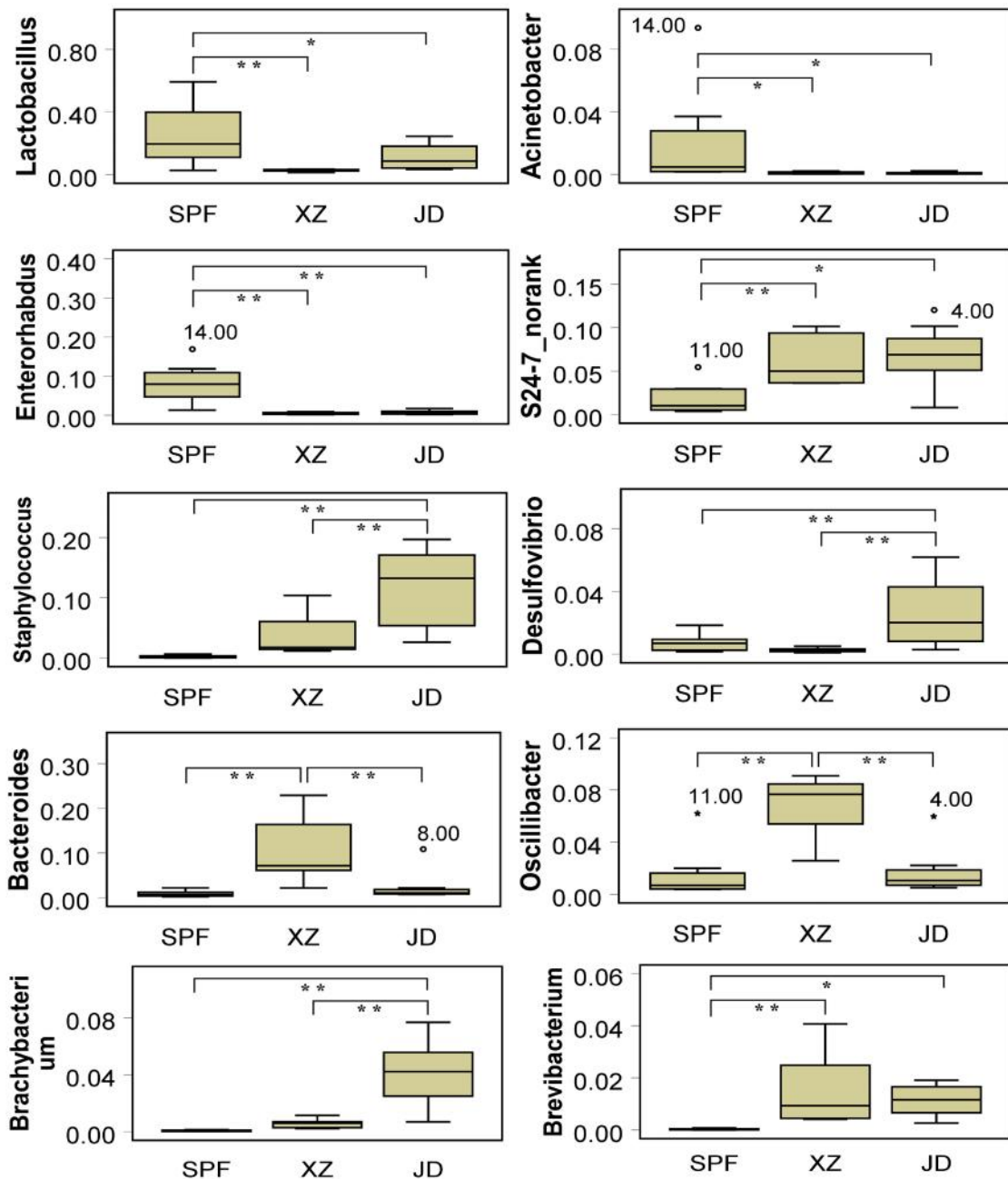


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1 Fig. S2. Top 10 most abundant genera in the murine gut microbiome. Comparison
2 among groups SPF (control), Jiang Du (JD), and Xiao Zhuang (XZ; n = 8; * $P < 0.05$,
3 ** $P < 0.01$ according to two-tailed least significant difference test).

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1 Fig. S3. Diagram depicting genera that significantly differentiated ($P < 0.05$) the three
2 mouse groups: SPF (control), Jiang Du (JD) and Xiao Zhuang (XZ).

3 A. “+” indicates genera significantly more abundant in JD than XZ and SPF.

4 B. “+” indicates genera significantly more abundant in XZ than JD and SPF.

5 C. “+” indicates genera significantly more abundant in SPF than JD and XZ. “-”
6 indicates genera significantly less abundant in SPF than JD and XZ.

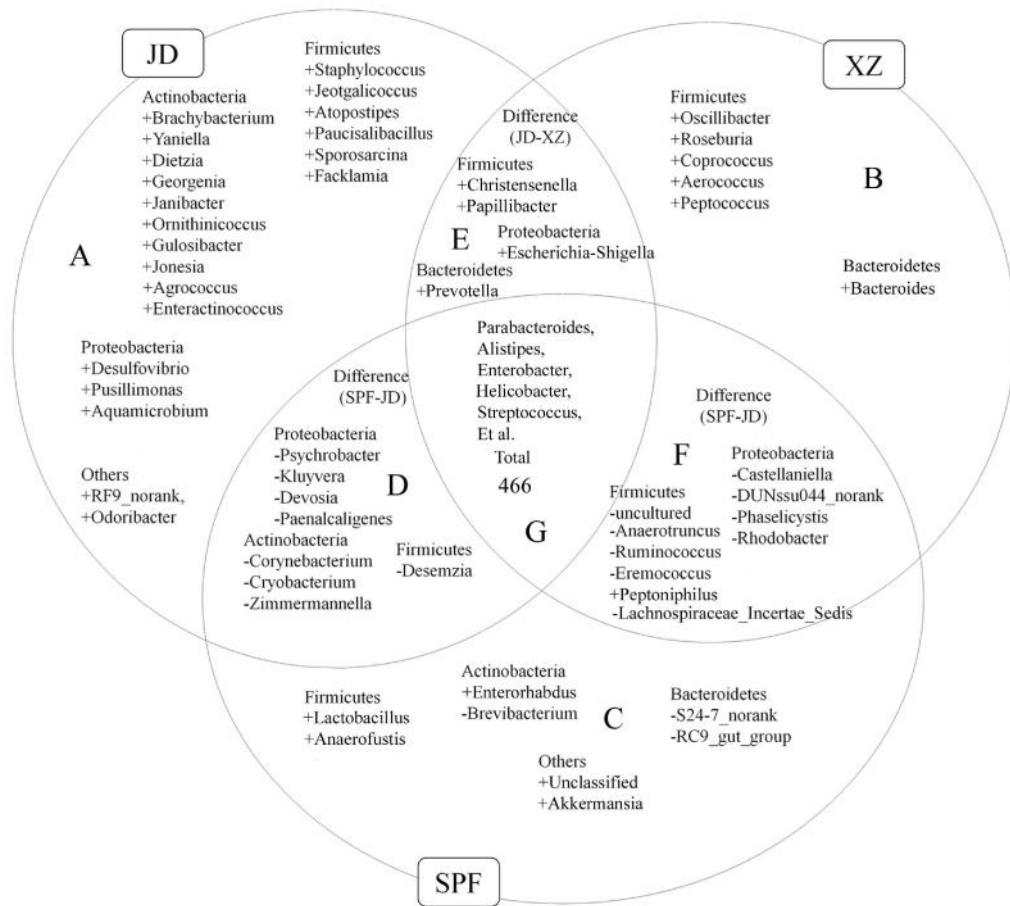
7 D. “-” indicates genera significantly less abundant in JD than SPF.

8 E “+” indicates genera significantly more abundant in JD than XZ.

9 F. “+” indicates genera significantly more abundant in SPF than XZ. “-” indicates
10 genera significantly less abundant in SPF than XZ mice.

11 G. Genera of equal abundance

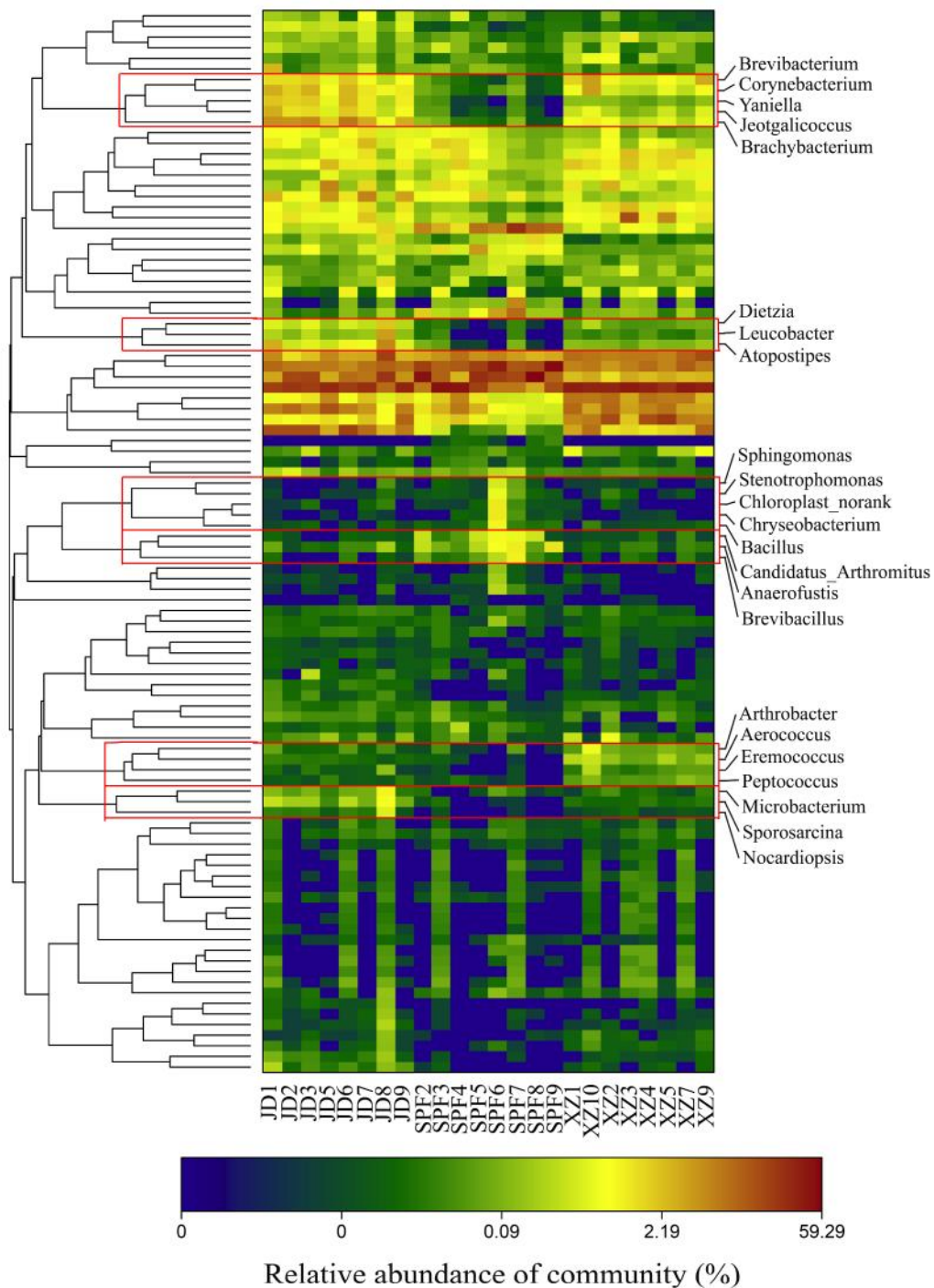
12 A total of 36 genera were significantly more abundant in JD than in XZ and/or SPF
13 mice, 18 genera were significantly more abundant in XZ than in JD and/or SPF mice,
14 6 genera were significantly more abundant in SPF than in JD and/or XZ mice, and
15 466 genera were equally abundant in JD, XZ and SPF mice.



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2

1 Fig. S4. Heat map profiles and dendrograms of top most abundant genera in gut
 2 microbiota for all samples. Mouse groups: SPF (control), Xiao Zhuang (XZ), and
 3 Jiang Du (JD). Color represents relative abundances.



- 1 Fig. S5. Dendrogram of complete-link hierarchical clustering. Mouse groups: SPF
- 2 (control), Xiao Zhuang (XZ), and Jiang Du (JD).

