

RESEARCH ARTICLE

Changes in gut microbiota composition with age and correlations with gut inflammation in rats

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Abstract

Increasing evidences indicate that gut microbiota composition is associated with multiple inflammatory diseases. However, little is known about how gut microbiota changes with age and correlations with gut inflammation at sexual maturity stage of healthy individuals. Elucidating the dynamic changes of gut microbiota in healthy individuals at the sexual maturity stage and correlations with gut inflammation can provide clues for early risk assessment of gut diseases at the sexual maturity stage. Here, the shift in gut bacteria and its relationship with gut inflammation at the sexual maturity stage were explored. Sprague–Dawley rats at the sexual maturity stage were used in this study. 16S rRNA gene sequencing was performed to decipher gut bacteria shifts from the 7th week to the 9th week, and enzyme-linked immunosorbent assay (ELISA) was used to measure gut inflammation and gut barrier permeability. We found an increase in bacterial richness with age and a decrease in bacterial diversity with age. The gut bacteria were primarily dominated by the phyla *Firmicutes* and *Bacteroides* and the genus *Prevotella*. The relative abundance of *Firmicutes* increased with age, and the relative abundance of *Bacteroides* decreased with age. There was a positive correlation between body weight and the *Firmicutes*:*Bacteroides* ratio. More and more gut microbiota participated in the host gut inflammation and barrier permeability regulation with age. *Ruminococcus* was the only gut bacteria participated in gut inflammation and barrier permeability regulation both in the 7th week and the 15th week. These results provide a better understanding of the relationship between gut bacteria and gut inflammation in sexually mature rats and show that *Ruminococcus* may be a potential indicator for early risk assessment of gut inflammation.

Introduction

Gut bacteria are considered to be a highly complex and diverse bacterial community that contributes to body health and disease [1,2]. When gut bacteria dysbiosis occurs, it will cause

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various health problems. For example, inflammatory bowel disease (IBD) has been shown to be associated with gut bacteria dysbiosis [1,3–5]. Gut dysfunction leads to impaired gut barrier integrity and abnormal signals in the vagus nerve [6–8]. Impaired gut barrier integrity leads to bacterial migration, which aggravates gut inflammation and peripheral inflammation [9–11]. Gut inflammation has been shown to induce some diseases, such as depression, cardiovascular disease and Parkinson's disease [12,13]. More than 300 million people suffer from depression, and two-thirds of them have suicidal ideation, which is the second leading cause of death among people aged 15–29 years [14].

With the development of high-throughput sequencing technology, the differences of gut bacteria between young and old people have been widely explored. For example, the dominant bacteria were *Firmicutes* in young men, while *Bacteroides* were the dominant bacteria in the elderly [15,16]. The relative abundance of class *Betaproteobacteria* was higher in the below65 age group, whereas the relative abundance of class *Gammaproteobacteria* was higher in the above65 age group [17]. Aging increases gut permeability, and translocation of lumen bacterial products has been reported [18,19]. In addition, although gut microbiota composition has been widely reported to be associated with multiple inflammatory diseases, little is known about how gut microbiota changes and the relationship with gut inflammation at the sexual maturity stage of healthy individuals. Sexual maturity is a special stage, in which the endocrine and other physiological states of the body change greatly. Elucidating the dynamic changes of gut microbiota in healthy individuals at the sexual maturity stage and its relationship with gut inflammation is critical for understanding the clues of potential biological indicators that can be used for early risk assessment of gut diseases at sexual maturity stage.

In this study, we monitored the major shifts in fecal bacteria and their relationship to gut inflammation in sexually mature rats. Humans generally become sexually mature at approximately 22–28 years old, and rats become sexually mature at approximately 6 weeks old [20,21]. Therefore, repeated fecal sampling of rats was performed from 7 weeks until 15 weeks of age under controlled conditions, which corresponds to the sexually mature stage in rats. Our results provide new insight into the correlation between gut microbiota shifts and gut inflammation in sexually mature rats.

Materials and methods

Animals

Male Sprague–Dawley (SD) specific pathogen-free rats (150–160 g) were obtained from Beijing SPF Biotechnology Co., Ltd. (Beijing, China). All rats were housed under a 12 h light/dark cycle at a temperature of 21–22°C and a humidity of 55 ± 5%. All rats received the same sterile standard food (SFB Biotechnology Co. Ltd., Beijing, China) and tap water. All animal procedures involved in this study were performed in strict accordance with research guidelines for the care and use of laboratory animals and were approved by the ethics committee of Beihang University.

Experimental design

Briefly, six-week-old ($n = 6$) rats were allowed to adapt to the environment for one week prior to the experiment. Then, fresh feces were collected from each rat every week (9 weeks, 54 samples) and stored quickly at –80°C until analysis. The animal work presented in this study was approved by the ethics committee of Beihang University. The experimental workflow is summarized in Fig 1A.

Information analysis of the 16S rRNA gene

DNA extraction and detection. At least 1 g of homogeneous fresh fecal sample was collected in a sterile sample collection tube containing phosphate buffered saline (PBS) and stored quickly at -80°C until analysis. When all samples were collected, DNA extraction was performed [22,23]. First, fecal samples were thawed on ice, and DNA was extracted by the phenol/trichloromethane/isoamyl alcohol method [24]. The extract was treated with DNase-free RNase to eliminate RNA contamination. The quality of DNA and the DNA concentration were determined by a Nanodrop spectrophotometer (Thermo Scientific, MA, USA). The molecular size of DNA was determined by agarose gel electrophoresis. According to the concentration, DNA was diluted to $1\text{ ng}/\mu\text{L}$ using sterile water.

Amplicon generation. 16S rRNA genes (V3-V4) were amplified using 341F (5'-CCTAYG GGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') primers. All PCRs were carried out with $15\ \mu\text{L}$ of PCR Master Mix, $0.2\ \mu\text{M}$ forward and reverse primers, and $10\ \text{ng}$ of template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s. Finally, the reaction was held at 72°C for 5 min.

DNA library construction and sequencing. Sequencing libraries were generated using a DNA PCR-Free Sample Preparation Kit (Illumina, CA, USA) following the manufacturer's recommendations. The library quality was assessed on a fluorometer (Thermo Scientific, MA, USA) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina NovaSeq platform, and 250 bp paired-end reads were generated.

Microbiota analysis. The 16S rRNA gene sequences were processed using R software (Version 4.0.0, <https://cran.r-project.org>), USEARCH 10.0 (<http://www.drive5.com/usearch/>) and in-house scripts [25]. Data were visualized by using Rstudio (Version 1.3.959.0, <https://rstudio.com/>) and ImageGP (<http://www.ehbio.com/ImageGP>). Barcodes (S1 Table) and primer sequences were

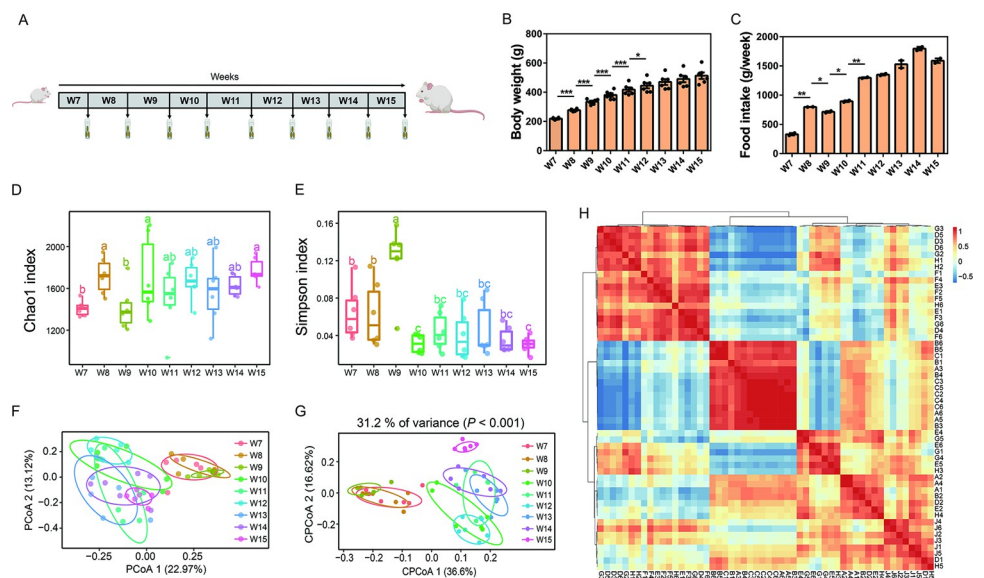


Fig 1. Diversity of gut bacteria shifts with time. (A), Experimental design and procedures. (B-C), Body weight and food intake shift with time ($n = 6$). (D-E), Chao 1 and Simpson indices were plotted to evaluate and compare α -diversity ($n = 6$). For boxplot with the same letter, the difference is not statistically significant. For boxplot with different letters, the difference is statistically significant. (F-G), Principal coordinate analysis and constrained principal coordinate analysis were plotted to evaluate and compare β -diversity ($n = 6$). (H), Hierarchical clustering using weighted UniFrac distances. OTU abundance levels were used to construct the heatmap ($n = 6$). Letters A-J represent the 7th week to 15th week, and numbers 1–6 represent six rats. Data were represented as means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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truncated. Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic units (OTUs). Representative sequences for each OTU were screened for further annotation. Representative sequences of OTUs were selected and analyzed with the USEARCH and Greengenes databases (Greengenes V13.5, http://www.drive5.com/sintax/gg_16s_13.5.fa.gz) to annotate taxonomic information: Kingdom, Phylum, Class, Order, Family, Genus and Species. The OTU table was standardized to the sample with the lower number of sequences and subjected to the following analyses.

Alpha diversity was used to analyze species diversity, including Chao1 and Simpson. Chao1 was selected to identify community richness, and Simpson was selected to identify community diversity. Tukey's test and the LSD test were used to analyze differences and visualized by Rstudio (Version 1.3.959.0, <https://rstudio.com/>). Beta diversity was used to analyze the differences between samples in terms of species complexity and visualized by Rstudio (Version 1.3.959.0, <https://rstudio.com/>), including principal coordinate analysis (PCoA) analysis and constrained principal coordinate analysis (CPCoA). PCoA and CPCoA were performed to obtain principal coordinates and visualize complex, multidimensional data. A distance matrix was transformed to a new set of orthogonal axes, by which the maximum variation factor was demonstrated by the first principal coordinate, and the second maximum variation factor was demonstrated by the second principal coordinate. The *vegan*, *ggplot2*, *reshape2*, *digest*, *ggrepel* and *ggpubr* packages of R software were used in PCoA analysis. The *Reshape2*, *ggplot2*, *vegan*, *digest*, *ggrepel* and *ggpubr* packages of R software (Version 4.0.0, <https://cran.r-project.org>) were used in CPCoA analysis.

BugBase (<https://bugbase.cs.umn.edu/>) is a microbiome analysis tool that determines phenotypes present in microbiome samples. The OTU table is the input file (S2 Table) in BugBase analysis. Briefly, a standardized OTU table was input, and then the microbial phenotype was predicted by the BugBase tool. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (<http://picrust.github.io/picrust/>) was performed using the online version of Galaxy (<http://huttenhower.sph.harvard.edu/galaxy/>). 16S rRNA data in the form of a BIOM format table were selected by mapping all 16S rRNA reads to references in the Greengenes tree with OTUs assigned at 97% identity. The obtained OTU table was normalized by the 16S rRNA gene copy number, and metagenomes were predicted from the KEGG catalog.

Cytokine measurements

Fecal samples were homogenized with phosphate-buffered saline (PBS) (60 Hz, 5 mins), and 1 g of each fecal sample was homogenized with 1 mL of PBS (1:1). White corundum was used as homogenate medium. After centrifugation at 4°C and 2800 g for 10 minutes, the fecal supernatant was extracted and stored at -80°C until analysis. A rat enzyme-linked immunosorbent kit (Beijing RGB Technology Development Co., Ltd.) was applied to measure the levels of the inflammatory cytokines TNF- α , IFN- γ , IL-6, IL-1 β , corticosterone (CORT), IL-10 and lipopolysaccharide (LPS), gut barrier permeability indicators iFABP and Zonulin according to the manufacturer's instructions. Briefly, samples were thawed, vortexed, centrifuged, incubated, and measured by ELISA (Thermo Multiskan MK3, Finland).

Correlation analysis

Correlations between the relative abundance of individual bacteria at the genus level and cytokine expression levels were analyzed using Spearman's rank correlation coefficient, and data were visualized by Rstudio.

Statistical analysis

Prior to determining the significance with parametric tests, normality was tested using the D'Agostino-Pearson omnibus normality test. For normally distributed data, significance was

determined using an unpaired two-tailed Student's *t*-test. The Mann-Whitney test was used when data failed the normality test (GraphPad Software Version 6.01, Inc., La Jolla, CA, USA). Histograms and line charts are presented as the means \pm standard error (SE). $P < 0.05$ was considered to indicate a statistically significant difference. Differences were noted as significant * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical analysis was carried out using Prism software (GraphPad Software Version 6.01, CA, USA) and OriginPro (Origin Software Version 2018C, MA, USA).

Results

Growth state and food intake

In this study, body weight and food intake were assessed every week. Compared with the previous week, body weight increased significantly from the 8th week to the 12th week ($P < 0.05$), but no significant weight gain was observed from the 13th week to the 15th week ($P > 0.05$) (Fig 1B). The average food intake increased in the 8th, 10th and 11th week, but decreased in the 9th week (Fig 1C).

Gut bacterial diversity shifts with time

We tracked fecal samples from rats to explore gut bacterial shifts over time. A total of 2,492,337 high-quality sequences were obtained from 54 samples (ranging from 26,923 to 61,584 reads per sample). We discarded low abundance OTUs and obtained 7,242 OTUs that could be used for further analysis. Alpha diversity analysis showed that the richness (Chao1 index) of gut bacteria significantly increased with time, while the diversity (Simpson index) of gut bacteria significantly decreased with time (Fig 1D and 1E). PCoA and CPCoA were used to visualize the shifts of beta diversity with time. According to the coordinate analysis, the points of the 7th week to the 9th week were clustered together, while the points of the 10th week to the 15th week were clustered together. In addition, the 7th week to 9th week and 10th week to 15th week were obviously divided into different groups on the PC1 axis (Fig 1F and 1G), indicating that the diversity of gut bacteria shifted with time.

To identify the similarities and differences of bacterial communities at different time points, hierarchical clustering was implemented (Fig 1H). Consistent with the results of PCoA and CPCoA, two different clusters were observed between the 7th week and the 9th week and between the 10th week and the 15th week, indicating that bacterial diversity at different ages showed large shifts.

Major shifts in the composition of gut bacteria

At the phylum level, the most predominant bacterial phyla were *Firmicutes* and *Bacteroides* (Fig 2A and 2B). At the genus level, *Prevotella* was the predominant genus (Fig 3A and 3B). A significant difference was observed between the 7th week to the 9th week and between the 10th week to the 15th week at the phylum level ($P < 0.01$) (Fig 2B). The relative abundance of *Firmicutes* increased significantly from the 10th week to the 15th week, while the relative abundance of *Bacteroides* decreased significantly from the 10th week to the 15th week (Fig 2C and 2D). However, the *Firmicutes/Bacteroides* (F/B) ratio was increased from the 10th week (Fig 2E). In addition, a positive correlation between body weight and the F/B ratio was observed ($r = 0.5$, $P < 0.001$) (Fig 2F).

Then, we analyzed the relative abundance and shifts of potential anti-inflammatory bacteria and pro-inflammatory bacteria (Fig 3C–3L). Potential anti-inflammatory bacteria *Faecalibacterium*, *Lactobacillus*, *Phascolarctobacterium* and *Prevotella* showed obvious shift ($P < 0.05$)

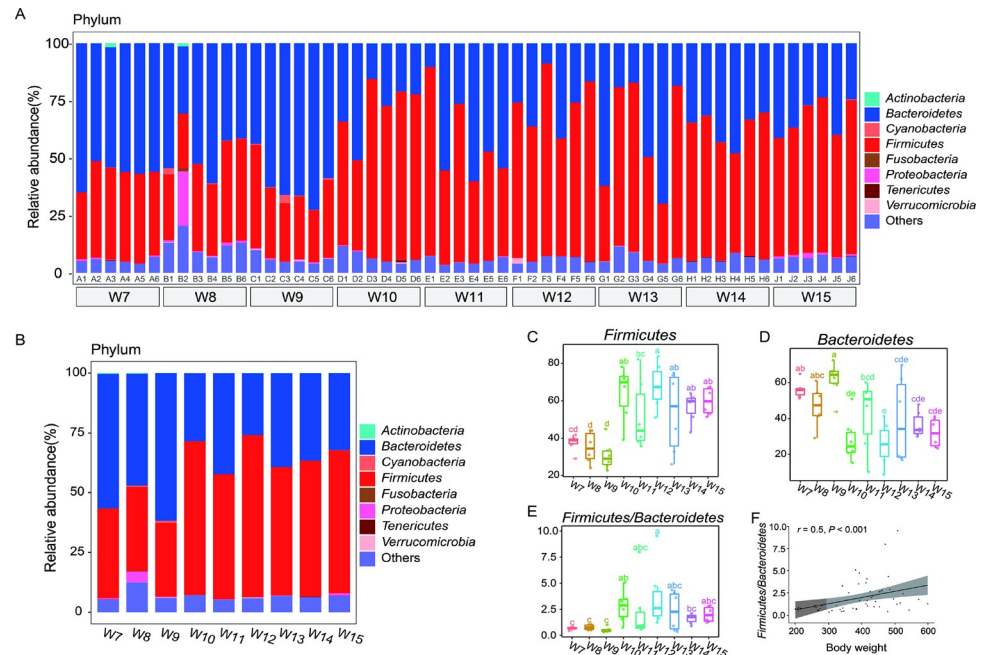


Fig 2. Gut bacteria composition at the phylum level. (A-B), Gut bacterial composition at the phylum level was compared for each sample and each group (n = 6). (C-E), The relative abundance of *Firmicutes* and *Bacteroidetes* shifted with time (n = 6), and the *Firmicutes*:*Bacteroidetes* ratio shifted with time (n = 6). For boxplot with the same letter, the difference is not statistically significant. For boxplot with different letters, the difference is statistically significant. (F), Spearman correlation between body weight and the *Firmicutes*:*Bacteroidetes* ratio (n = 54).

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(Fig 3D–3L). Although potential anti-inflammatory bacteria *Lachnospiraceae* showed fluctuation, the overall fluctuation was relatively stable ($P = 0.5$). *Faecalibacterium* increased greatly in the 8th week, while *Lactobacillus* and *Phascolarctobacterium* increased greatly from the 9th week to the 13th week. Potential pro-inflammatory bacteria *Bacteroides*, *Turricibacter*,

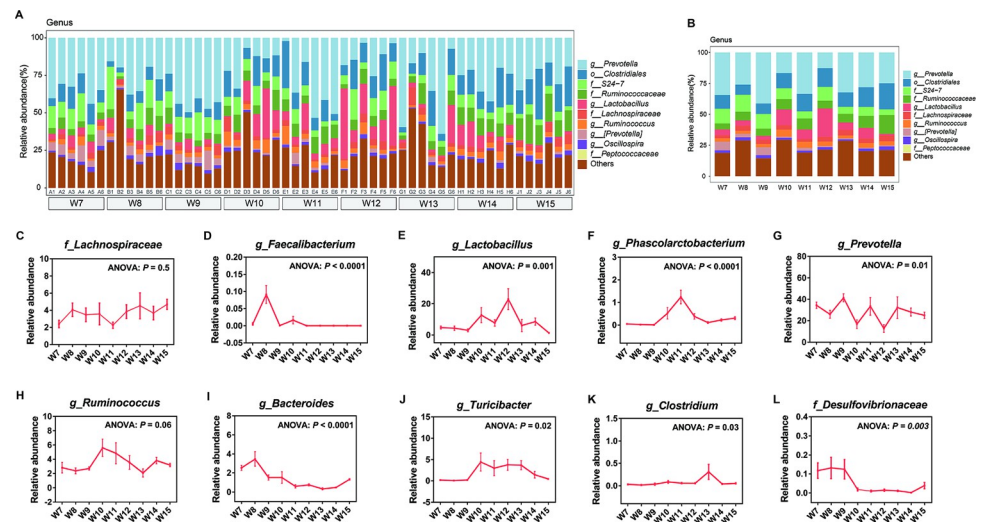


Fig 3. Gut bacteria composition at the genus level. (A-B), Gut bacterial composition at the genus level was compared for each sample and each group (n = 6). (C-L), Certain genera shifted with time (ANOVA test followed by post-hoc tests, n = 6).

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Clostridium and *Desulfovibrionaceae* showed a decreasing trend ($P < 0.05$), among which the relative abundance of *Bacteroides* and *Desulfovibrionaceae* showed an obvious downward trend from the 9th week to the 15th week (Fig 3I–3L).

Gut bacteria are associated with gut inflammation

As shown in Fig 4, we measured the levels of multiple cytokines (TNF- α , IFN- γ , IL-6, CORT, IL-1 β , IL-10, LPS, iFABP and Zonulin). Compared with the 7th week, TNF- α , IFN- γ , IL-6, CORT, IL-1 β , IL-10, LPS and Zonulin concentrations were decreased in the 15th week, but no significant differences in any of these cytokines were observed (Fig 4A–4I).

Spearman correlation analysis was used to calculate the relationship between the relative abundance of gut bacteria and cytokine levels (Fig 5A and 5B). We found that *Ruminococcus* was significantly negatively correlated with IFN- γ in the 7th week ($r = -0.8$, $P < 0.05$) and significantly positively correlated with iFABP in the 15th week ($r = 0.9$, $P < 0.05$). In addition, *Clostridiales* ($r = 0.9$, $P < 0.01$) and *Lactobacillus* ($r = 1$, $P < 0.001$) were significantly positively

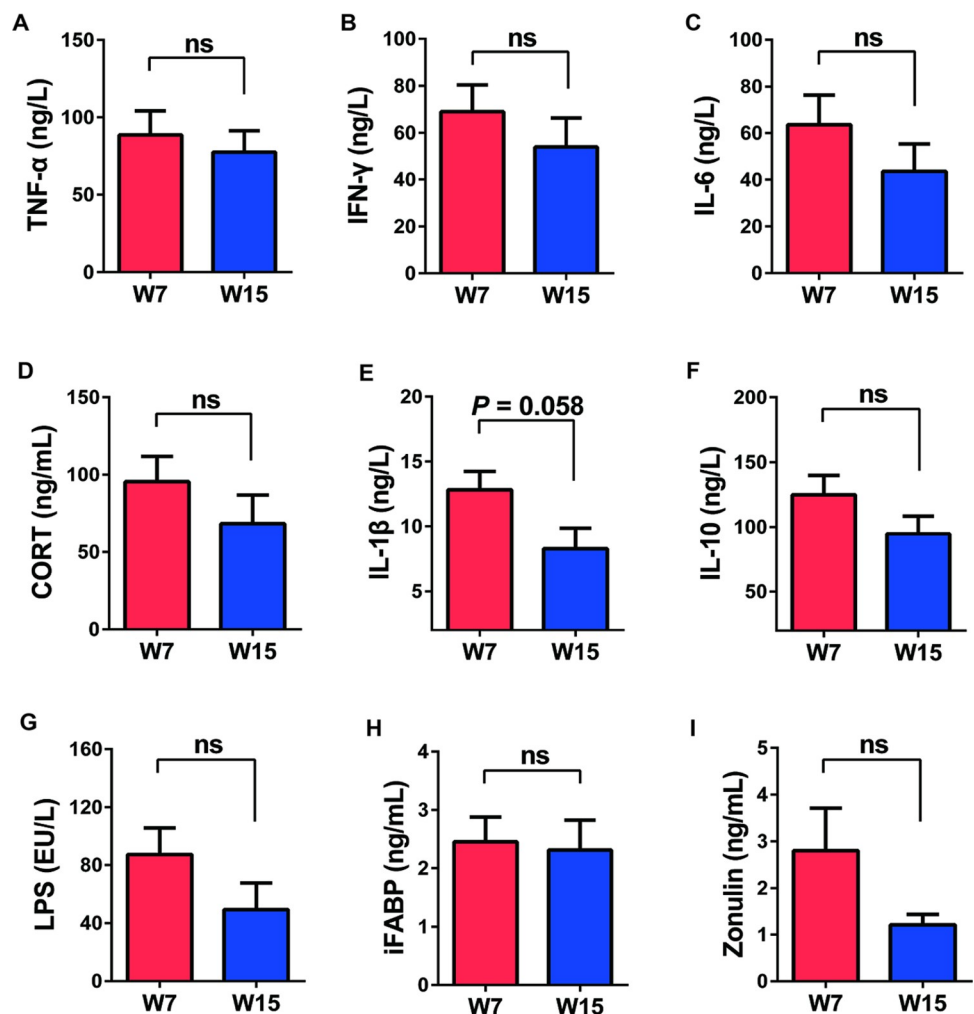


Fig 4. Gut inflammation, bone metabolism and gut barrier permeability-related cytokine expression in sexually mature rats. (A–G), Levels of inflammation-associated cytokines were measured in fecal samples ($n = 6$). (H–I), Levels of gut barrier permeability-associated cytokines were measured in fecal samples ($n = 6$). Data were represented as means \pm SEM. ns, no significance.

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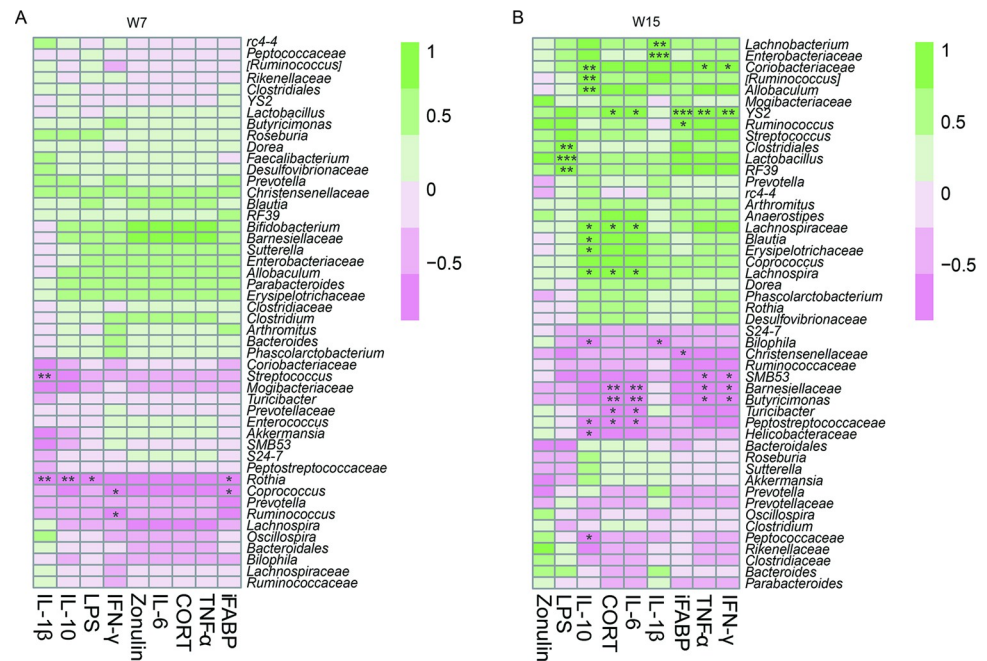


Fig 5. Spearman correlation between gut bacteria and gut inflammation. (A), Spearman correlation between gut bacteria and gut inflammation and gut barrier permeability-related cytokine expression in the 7th week. (B), Spearman correlation between gut bacteria and gut inflammation and gut barrier permeability-related cytokine expression in the 15th week. Note: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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correlated with LPS in the 15th week. *Peptococcaceae* was significantly negatively correlated with IL-10 in the 15th week ($r = -0.8$, $P < 0.05$). *Lachnospiraceae* was significantly positively correlated with CORT ($r = 0.9$, $P < 0.05$), IL-6 ($r = 0.9$, $P < 0.05$) and IL-10 ($r = 0.8$, $P < 0.05$) in the 15th week. Compared with the 7th week, the gut bacteria that correlated with gut inflammation and barrier permeability increased significantly in the 15th week, suggesting that an increasing number of gut bacteria were involved in host gut inflammation and barrier permeability regulation.

Further insight into bacterial functions

Compared with the 7th week, the relative abundance of the anaerobe phenotype showed no significant shift, and the relative abundance of the potentially pathogenic phenotype decreased with time ($P < 0.05$), suggesting that host gut bacteria were developing toward healthier states (Fig 6A and 6B).

The metabolic pathway activities of environmental information processing, genetic information processing and metabolism increased with time (Fig 6C). ABC transporters, bacterial toxins, tyrosine metabolism and lipid metabolism pathway activities were significantly increased with time ($P < 0.05$) (Fig 6D, 6E, 6H and 6J). DNA repair and recombinant protein pathway activities showed an increased trend ($P = 0.06$) (Fig 6F). The activities of lipopolysaccharide biosynthesis, ubiquinone and other terpenoid quinone biosynthesis, and xanthine dehydrogenase iron-sulfur-binding subunit metabolic pathway activities were significantly decreased with time ($P < 0.001$) (Fig 6G, 6I and 6K). Meanwhile, there was an obvious distinction in metabolic pathway activity between the 7th week and the 9th week and between the 10th week and the 15th week (Fig 6D–6K).

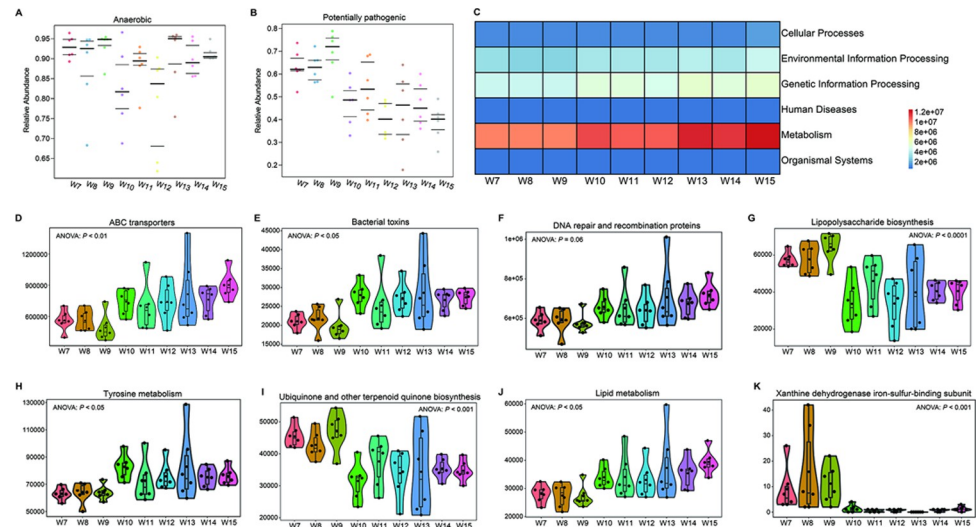


Fig 6. Bacterial gene function annotation and phenotype prediction. (A-B), Anaerobic and potentially pathogenic shifts with time are shown. (C-K), The average abundance of differentially enriched KEGG pathways (ANOVA test followed by post-hoc tests).

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Discussion

Gut bacterial development is a crucial factor affecting host inflammation, immunity and disease [26,27]. However, a study on the functional characterization of gut bacterial strains has not been conducted, and the role of bacteria in different diseases is not completely consistent. We found that the gut bacterial community shifted in a successional manner, and the bacterial community developed with increasing richness and decreasing diversity with age.

Lachnospiraceae was decreased in children with diarrhea, and supplementation with *Lachnospiraceae* alleviated obesity and inflammation in mice [28,29]. In this study, we found that *Lachnospiraceae* was positively associated with the levels of the anti-inflammatory cytokine IL-10, suggesting that *Lachnospiraceae* may play positive roles in host gut inflammation. *Ruminococcus* was negatively associated with pro-inflammatory cytokine IFN- γ levels and positively associated with iFABP, suggesting that *Ruminococcus* may also play positive roles in host gut inflammation. We found that *Lactobacillus* was strongly positively associated with LPS levels, an endotoxin that promotes the synthesis and release of various cytokines and inflammatory mediators. Greatly increases in *Lactobacillus* abundance from the 9th week to 13th week may imply that the body suffers from some abnormal changes and more *Lactobacillus* were needed. In addition, *Lachnospiraceae*, *Faecalibacterium*, *Ruminococcus* and *Lactobacillus* were considered to be related to the expression of short-chain fatty acids (SCFAs) [30–33]. Studies have suggested that SCFAs have an important role in host metabolism, such as improving post-stroke recovery, reducing the development of severe pancreatic insulinitis, alleviating psychological problems and influencing the gut-brain axis [34–39]. *Bacteroides*, *Desulfovibrionaceae* and *Turricibacter* were considered to have potential proinflammatory properties in some studies [40–44]. However, we found that *Turricibacter* was significantly negatively correlated with the proinflammatory cytokine IL-6, suggesting that *Turricibacter* may have potential anti-inflammatory properties. There was a decreasing trend of gut inflammation over the sexual maturity stage in this study, which was consistent with the trend of LPS biosynthesis pathway activity in functional prediction.

In addition, it is worth noting that the results of this study were based on male rats. There is always controversy about whether male or female rats should be used in the experiment. Both female and male rats are affected by hormones, but males change more in one day, while females change more in the long term and have more variables [45]. Females and males change through different ways and different time scales. It is worth noting that the differences in physiological structure, hormone secretion level and secretory cycle between female and male rats may lead to the differences in research results.

In summary, we tracked the changes in gut bacteria and gut inflammation with growth. We found a gradual increase in bacterial richness with age and a gradual decrease in bacterial diversity with age. More and more gut microbiota participated in the host gut inflammation and barrier permeability regulation with age, and *Ruminococcus* was the only gut bacteria participated in gut inflammation and barrier permeability regulation both in the 7th week and the 15th week in this study. These results provide a better understanding of the relationship between gut bacteria and gut inflammation with age in sexually mature rats.

Ethical approval

All procedures were performed according to the National Institutes of Health Guide for the care and use of laboratory animals, rats were euthanized with carbon dioxide and were approved by the ethics committee of Beihang University.

Supporting information

S1 Table. Barcodes and primer sequences information.
(XLSX)

S2 Table. The normalized OTUs in BugBase analysis.
(XLSX)

Author Contributions

Conceptualization: Chen Meng, Siyuan Feng, Chen Dong, Hong Liu.

Data curation: Chen Meng, Hong Liu.

Formal analysis: Chen Meng, Siyuan Feng, Zikai Hao, Chen Dong, Hong Liu.

Funding acquisition: Hong Liu.

Investigation: Chen Meng, Chen Dong, Hong Liu.

Methodology: Chen Meng, Zikai Hao, Chen Dong, Hong Liu.

Project administration: Chen Meng.

Software: Chen Meng, Zikai Hao.

Supervision: Chen Dong, Hong Liu.

Validation: Chen Meng, Chen Dong, Hong Liu.

Visualization: Chen Meng, Zikai Hao, Chen Dong, Hong Liu.

Writing – original draft: Chen Meng, Zikai Hao, Chen Dong, Hong Liu.

Writing – review & editing: Siyuan Feng, Chen Dong, Hong Liu.

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