Lysozyme Treatment Makes 16S rRNA Amplicon Sequencing Results Less Biased

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Research Article

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Abstract

Background

Amplicon sequencing is widely applied in gut bacteria structure analysis. However, the proportion of Gram-positive bacteria may greatly affect the results of microbial community analysis. Lysozyme is an effective agent to extract DNA of Gram-positive bacteria. In this study, we assessed the influence of lysozyme treatment on results of *Bactrocere dorsalis* rectal bacteria structure.

Result

The results indicated that the total bacteria content can be significantly increased in lysozyme treated samples. Moreover, rectal bacteria diversity was significantly higher in lysozyme treated samples. A detail analysis revealed that abundance of Gram-positive bacteria significantly increased in samples treated with lysozyme.

Conclusion

This study indicates that lysozyme treatment before DNA extraction is an effective way to reduce bias in bacteria structure analysis, especially for samples with high proportion of Gram-positive bacteria.

Background

The structure of gut bacteria can be shaped by various factors[1]. Diet can shape the gut bacteria of human [2]. On the contrary, gut bacteria can also affect the metabolism and behavior of their hosts [3, 4]. Studies have indicated that even obesity and lean of human can be affected by the gut bacteria [5, 6]. With the development of sequencing technique, cost of 16s rRNA amplicon sequencing is dramatically reduced and functions of more and more gut bacteria are investigated in different hosts [7]. Insects and other terrestrial arthropods are closely associated with symbiotic microorganisms [8–10]. Among these special hosts, gut bacteria are pervasive. Thanks to the development of the 16s rRNA amplicon sequencing method, breakthroughs are made by identifying and classifying the gut bacteria of more and more insect hosts [11].

Previous study has shown that gut bacteria sequencing results may be affected by various factors [12–14]. DNA extraction method is one of the most vital factors that can influence the sequencing results besides the primers and sequencing platform [15]. Normal extracting steps may lead to so huge bias on the result of sequencing that it leads to completely different conclusions. For example, some studies have showed that Proteobacteria (most are Gram-negative bacteria) occupied the dominant status in *B. dorsalis* guts, while others have claimed that the Firmicutes (most are Gram-positive bacteria) is the major class [5, 16]. Such difference may be caused by Gram-positive bacteria, since different DNA

extraction method can result in a huge difference in abundance of Gram-positive bacteria. Therefore, it is urgent to optimize the method of DNA extraction to reduce the bias in gut bacteria analysis.

With the invention of Gram staining, bacteria can be divided into two groups by the staining color, Gram-positive and Gram-negative bacteria [17, 18]. The cell wall structures of the Gram-positive and Gram-negative bacteria are totally different. For Gram-positive bacteria, the cell wall is thicker with more layers of membrane and peptidoglycan and less lipid. Lysozyme can destroy the β-1,4-glycosidic bond between n-acetyl intracytoic acid and N-acetyl glucosaccharide which is an important component in the cell wall of Gram-positive bacteria. With β-1,4-glycosidic being destroyed, the insoluble mucosaccharide in cell wall can be decomposed into soluble glycopeptides, resulting in the rupture of cell wall and the escape of the contents and finally causing the dissolution of bacteria [19, 20]. Therefore, it is an effective way to extract the DNA of Gram-positive bacteria by incubating with lysozyme.

In this study, we analyzed the 16s rRNA amplicon sequencing results of *B. dorsalis* rectum bacteria for which DNA was extracted by Bacterial genome kits adding with lysozyme or not. The results showed that the rectal bacteria diversity and Gram-positive bacteria abundance in lysozyme treatment was significantly higher than none-lysozyme treatment.

Results

Lysozyme incubation affects the total bacteria content extracted from rectum

qPCR was used to test if the total bacteria content in rectum can be affected by lysozyme incubation. The results showed that total bacteria content could be significantly affected by lysozyme incubation. For newly emerged male, bacteria content in rectum was significantly higher if the DNA was extracted without lysozyme treatment. However, bacteria contents were significantly lower if the DNA was extracted without lysozyme treatment in 3- and 6-day-old male. For 9- and 12-day-old male, there was no difference for total bacteria contents (Figure 1).

Rectum bacteria diversity was significantly affected by lysozyme incubation

To test if lysozyme incubation would affect bacteria diversity, 16S rRNA amplicon sequencing was used to investigate rectum bacteria diversity. We assessed the impact of lysozyme incubation on sequencing data. The results showed that both sequencing tag and OTU numbers were significantly higher in lysozyme incubation samples (Figure 2A and 2B). Furthermore, α diversity and β diversity represented by Shannon indices and PCoA analysis were assessed. The results were in agreement with the sequencing data evaluation results: Shannon indices were significantly higher in lysozyme incubation samples (Figure 2C). PCoA plots of non-lysozyme treatment samples showed that the rectum samples collected at different time points were highly clustered, while distinct separations were showed in lysozyme treatment samples (Figure 2D and 2E). These results indicate lysozyme treatment before DNA extraction is vital for investigating the bacteria diversity in rectum accurately.

Lysozyme treatment significantly affected the taxonomic results

Taxonomic compositions of the rectum bacteria were compared to test whether lysozyme treatment can affect the bacteria community composition. For lysozyme treated samples, rectum bacteria changed significantly at different time points. The dominant taxa in 0d samples was Gammaproteobacteria and Bacilli, while Bacilli was the only dominant taxa in 3d, 6d, 9d, and 12d samples. However, bacteria composition patterns were significantly different in non-lysozyme treated samples. Gammaproteobacteria, Bacilli and Clostridia were the dominant taxa in rectum at all the time points and the abundance of the same bacteria group fluctuated greatly in the repeated samples, which indicated that DNA extraction efficiency of the rectum bacteria was very unstable (Figure 3).

Since lysozyme can effectively improve DNA extraction efficiency of Gram-positive bacteria [20], We divided the rectum bacteria into two groups (Gram-negative and Gram-positive bacteria) and compared their abundance. In the top six groups in term of abundance, Bacilli, Clostridia and Actinobacteria were mainly Gram-positive bacteria, while Gammaproteobacteria, Bacteroidia and Alphaproteobacteria were mainly Gram-negative bacteria (Figure 3). For 0d rectum samples, none of Gram-positive and Gram-negative bacteria were significantly different in abundance between samples treated with lysozyme and control (Figure 4). However, Gram-positive bacteria abundance were significantly higher in samples treated with lysozyme for 3d, 6d, 9d and 12d rectum and the abundance of Gram-negative bacteria were significantly lower (Figure 4). These results indicated that lysozyme can significantly increase the abundance of Gram-positive bacteria extracted in rectum.

Discussion

In this study, bacteria content and structure in *B. dorsalis* rectum were compared between rectum DNA extracted with lysozyme treatment and none-lysozyme treatment. Our results indicated that lysozyme treatment before DNA extraction could significantly increase the extracted bacteria content and broad the range of the rectal bacteria communities.

Lysozyme treatment before DNA extraction could significantly increase the content of bacteria in *B. dorsalis* rectum at the early stages after eclosion. With the development of the host insects, the gut bacteria abundance may be affected by metabolism of host insect [21]. Studies have indicated that the bacteria content of insect is relatively low at the early stages after elosion [21, 22]. Thus, the bacteria content may be significantly lower in rectum for which Gram-positive bacteria were dominant and DNA was extracted without lysozyme treatment. Though the bacteria content showed no difference at the later stages, the bacteria structures were significantly different. Physically grinding and thermal incubation could increase the efficiency of DNA extraction, but it wasn't enough to destroy the cell wall of Gram-positive bacteria entirely [23]. Thus, extracting DNA without lysozyme treatment may lose the Gram-positive bacteria, which may lead to bias in bacteria structure [24].

In *B. dorsalis* rectum, Bacilli, Gammaproteobacteria and Alphaproteobacteria were three dominant taxa in the lysozyme treatment while most bacteria in none-lysozyme treatment rectum were

Gammaproteobacteria. Though some Gram-positive bacteria could be identified in none-lysozyme treatment samples, their abundance was significantly lower than the lysozyme treatment. Without lysozyme treatment, the abundance of Gram-positive bacteria had great fluctuation within the same groups. Thus, those Gram-positive bacteria may be one of the factors that resulted in bias in the sequencing results.

Recent years, the 16s rRNA amplicon sequencing technology have become a fundamental technology in bacteria community analysis. Reflecting a real composition and diversity of the bacteria is the basis for analyzing the function of the related bacteira [25–27]. To reduce the bias of sequencing results, researchers continued to optimize the DNA extraction method before sequencing in different dimension including the extraction protocols, sampling methods and surface materials in different cases [7, 28]. Some researchers had noticed the importance of lysozyme used in the DNA extraction before sequencing, while others relied solely on the DNA extraction for the convenient, which could lead to a dramatic difference in bacteria communities [29–31].

Conclusion

Our research had showed that lysozyme treatment before DNA extraction was rather necessary to obtain the accurate information. Combining lysozyme treatment and DNA extraction kit together can reach a more complete composition of the bacteria, especially for those samples in which Gram-positive bacteria abundance was high.

Methods

Rectal sample collection

Male adult rectums (0, 3, 6, 9 and 12 days old) were dissected and soaked in absolute ethyl alcohol, respectively. Eight rectums were dissected and put into one centrifuge tube as one sample, 10 samples were collected for each stage (0, 3, 6, 9 and 12 days old flies). Then the samples were divided into two groups, one group containing 5 samples were sent for DNA extraction with Lysozyme treatment, the other group was used for DNA extraction without lysozyme treatment.

DNA extraction

The rectal DNA was extracted with the Bacterial Genome Extraction Kit (Tiangen, Beijing, China). Briefly, the samples in the centrifuge tubes were grinded in a grinder. Then one group of grinded samples was incubated in 180 μl lysozyme (20 mg/ml) for 50 minutes under 37°C. Then the samples were used for DNA extraction with the Bacterial Genome Extraction Kit following the manufacturer's protocols. The other group of samples without lysozyme incubation was used as control.

Total bacteria content quantification

The total bacteria content in the collected rectal samples was evaluated by real-time quantitative PCR. The 16S rRNA gene fragment was amplified using the universal primer of bacteria: 338F (5'-ACTCCTACGGGAGGCAGCAG -3') and 518R(3'-GGTCGTCGGCGCCATTA-5') [32]. Then the genomic DNA of *E. coli* was extracted for amplification with the primers. The amplified fragment was then cloned into the pMD 18–T vector, which was then transferred into *E. coli* DH5α to reproduce. The reproduced vector was then extracted with a plasmid extraction kit, and diluted in a series of 10-fold dilutions to obtain 7 different plasmid concentrations (measured by Nanodrop spectrophotometer). A standard curve for qPCR was then generated by amplifying the 16S rRNA of the plasmid. The absolute abundance of bacteria in the rectal was determined by referring to the standard curve.

16S rRNA amplicon sequencing for rectal bacteria

V3-V4 region of the 16S rRNA gene were amplified by PCR using primers 341F□CCTACGGGNGGCWGCAG; 806R□GGACTACHVGGGTATCTAAT. PCR reactions were performed in triplicate 50 μL mixture containing 5 μL of 10×KOD Buffer, 5 μL of 2.5 mM dNTPs, 1.5 μL of each primer (5 μM), 1 μL of KOD Polymerase, and 100 ng of template DNA. Then the amplicons were extracted from 2% agarose gels and purified using the DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) and quantified using QuantiFluor-ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2×250) on an Illumina platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA669205). Then the sequenced raw reads for each sample were cleaned and analyzed according to the standards of our previous studies [33].

Declarations

Ethics approval and consent to participate

Ethical approval was not required for this work as no human or animal samples and/or data were analysed in this study. B. dorsalis used in this study were collected from a carambola (Averrhoa carambola) orchard in Guangzhou, Guangdong Province and reared in the laboratory under the following conditions: 25 ± 1 °C, 16:8 h light: dark cycle, 70–80% RH. Adult flies were fed with yeast and sugar mixture.

Consent for publication

Not applicable.

Availability of data and materials

Sequence data are available from the NCBI Short Read Archive. The accession numbers are PRJNA669205 (lysozyme treated samples) and PRJNA780089 (non-lysozyme treated samples) .

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YJ contributed to analyzing the data, performing literature research and writing—original draft. SG done investigation. ZG collecting the data. LR performed experiment and investigation. YL done methodology and supervision. DC was involved in data curation, formal analysis, manuscript revising, review and editing, project administration, funding acquisition, and supervision. All authors read and approved the final manuscript.

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Not applicable

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References

1. Hassan B, Siddiqui JA, Xu Y: **Vertically Transmitted Gut Bacteria and Nutrition Influence the Immunity and Fitness of** *Bactrocera dorsalis* **Larvae**. *Frontiers in Microbiology* 2020, **11**:596352.
2. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S *et al*: **Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa**. *Proc Natl Acad Sci U S A* 2010, **107**(33):14691–14696.
3. Qiao H, Keesey IW, Hansson BS, Knaden M: **Gut microbiota affects development and olfactory behavior in** *Drosophila melanogaster*. *Journal Of Experimental Biology* 2019, **222**(5):2397–2404.
4. Ren F-R, Sun X, Wang T-Y, Yao Y-L, Huang Y-Z, Zhang X *et al*: **Biotin provisioning by horizontally transferred genes from bacteria confers animal fitness benefits**. *Isme J* 2020, **14**(10):2542–2553.
5. Andongma AA, Wan L, Dong YC, Li P, Desneux N, White JA *et al*: **Pyrosequencing reveals a shift in symbiotic bacteria populations across life stages of** *Bactrocera dorsalis*. *Sci Rep* 2015, **5**:9470.
6. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE *et al*: **A core gut microbiome in obese and lean twins**. *Nature* 2009, **457**(7228):480-U487.
7. Teng F, Nair SSD, Zhu P, Li S, Huang S, Li X *et al*: **Impact of DNA extraction method and targeted 16S-rRNA hypervariable region on oral microbiota profiling**. *Sci Rep* 2018, **8**:16321.
8. Akami M, Andongma AA, Chen ZZ, Nan J, Khaeso K, Jurkevitch E *et al*: **Intestinal bacteria modulate the foraging behavior of the oriental fruit fly** *Bactrocera dorsalis* **(Diptera: Tephritidae)**. *PLoS One*

2019, **14**(1):e0210109.

9. Cheng D, Chen S, Huang Y, Pierce NE, Riegler M, Yang F *et al*: **Symbiotic microbiota may reflect host adaptation by resident to invasive ant species**. *PLoS Pathog* 2019, **15**(7):e1007942.
10. Raza MF, Wang YC, Cai ZH, Bai S, Yao ZC, Awan UA *et al*: **Gut microbiota promotes host resistance to low-temperature stress by stimulating its arginine and proline metabolism pathway in adult** *Bactrocera dorsalis*. *PLoS Pathog* 2020, **16**(4):29.
11. Shang SQ, Chen GX, Wu YD, Du LZ, Zhao ZY: **Rapid diagnosis of bacterial sepsis with PCR amplification and microarray hybridization in 16S rRNA gene**. *Pediatric Research* 2005, **58**(1):143–148.
12. Fouhy F, Deane J, Rea MC, O'Sullivan O, Ross RP, O'Callaghan G *et al*: **The Effects of Freezing on Faecal Microbiota as Determined Using MiSeq Sequencing and Culture-Based Investigations**. *PLoS One* 2015, **10**(3):e0119355.
13. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G *et al*: **Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons**. *Genome Res* 2011, **21**(3):494–504.
14. Kennedy NA, Walker AW, Berry SH, Duncan SH, Farquarson FM, Louis P *et al*: **The Impact of Different DNA Extraction Kits and Laboratories upon the Assessment of Human Gut Microbiota Composition by 16S rRNA Gene Sequencing**. *PLoS One* 2014, **9**(2):e88982.
15. Fouhy F, Clooney AG, Stanton C, Claesson MJ, Cotter PD: **16S rRNA gene sequencing of mock microbial populations- impact of DNA extraction method, primer choice and sequencing platform**. *BMC Microbiol* 2016, **16**:123.
16. Hadapad AB, Shettigar SKG, Hire RS: **Bacterial communities in the gut of wild and mass-reared** *Zeugodacus cucurbitae* **and** *Bactrocera dorsalis* **revealed by metagenomic sequencing**. *BMC Microbiol* 2019, **19**(1):282.
17. Leite Vicente CS, Ozawa S, Hasegawa K: **The composition of hindgut microbiota of** *Periplaneta japonica* **in the presence of thelastomatid parasitic nematodes**. *Nematological Research* 2018, **48**(1):19–26.
18. Lombogia CA, Tulung M, Posangi J, Tallei TE: **Bacterial Composition, Community Structure, and Diversity in** *Apis nigrocincta* **Gut**. *International Journal of Microbiology* 2020, **2020**:6906921-Article No.: 6906921.
19. Primo ED, Otero LH, Ruiz F, Klinke S, Giordano W: **The Disruptive Effect of Lysozyme on the Bacterial Cell Wall Explored by an in-Silico Structural Outlook**. *Biochemistry and Molecular Biology Education* 2018, **46**(1):83–90.
20. Salton MR: **The properties of lysozyme and its action on microorganisms**. *Bacteriological reviews* 1957, **21**(2):82–100.
21. Huang F, Zhao C-L, Lu Y-B: **Dynamics of Tremblaya princeps during individual development of its host** *Phenacoccus solenopsis* **(Hemiptera: Pseudococcidae)**. *Acta Entomologica Sinica* 2015, **58**(10):1140–1145.

22. Broderick NA, Lemaitre B: **Gut-associated microbes of** *Drosophila melanogaster*. *Gut Microbes* 2012, **3**(4):307–321.
23. Proctor LM, Chhibba S, McEwen J, Peterson J, Wellington C, Baker C *et al*: THE NIH HUMAN MICROBIOME PROJECT; 2013.
24. Xue H, Zhu X, Wang L, Zhang K, Li D, Ji J *et al*: **Gut Bacterial Diversity in Different Life Cycle Stages of** *Adelphocoris suturalis* **(Hemiptera: Miridae)**. *Frontiers In Microbiology* 2021, **12**:670383.
25. Breakwell K, Tetu SG, Elbourne LDH: **Next Generation Barcode Tagged Sequencing for Monitoring Microbial Community Dynamics**. In: *Environmental Microbiology: Methods and Protocols, 2nd Edition.* Edited by Paulsen IT, Holmes AJ, vol. 1096; 2014: 101-110.
26. Cannavan FS, Nakamura FM, Germano MG, de Souza LF, Tsai SM: **Next-Generation Sequencing to Elucidate Biochar-Effected Microbial Community Dynamics**; 2016.
27. Scholz MB, Lo C-C, Chain PSG: **Next generation sequencing and bioinformatic bottlenecks: the current state of metagenomic data analysis**. *Current Opinion in Biotechnology* 2012, **23**(1):9–15.
28. Maillet A, Bouju-Albert A, Roblin S, Vaissie P, Leuillet S, Dousset X *et al*: **Impact of DNA extraction and sampling methods on bacterial communities monitored by 16S rDNA metabarcoding in cold-smoked salmon and processing plant surfaces**. *Food Microbiol* 2021, **95**:103705.
29. Chen BS, Teh BS, Sun C, Hu SR, Lu XM, Boland W *et al*: **Biodiversity and Activity of the Gut Microbiota across the Life History of the Insect Herbivore** *Spodoptera littoralis*. *Sci Rep* 2016, **6**:29505.
30. Lanan MC, Rodrigues PAP, Agellon A, Jansma P, Wheeler DE: **A bacterial filter protects and structures the gut microbiome of an insect**. *Isme J* 2016, **10**(8):1866–1876.
31. Vogel H, Shukla SP, Engl T, Weiss B, Fischer R, Steiger S *et al*: **The digestive and defensive basis of carcass utilization by the burying beetle and its microbiota**. *Nat Commun* 2017, **8**:15186.
32. Liu W-T, Marsh TL, Cheng H, Forney LJ: **Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA**. *Applied and Environmental Microbiology* 1997, **63**(11):4516–4522.
33. Huang H, Ren L, Li H, Schmidt A, Gershenzon J, Lu Y *et al*: **The nesting preference of an invasive ant is associated with the cues produced by actinobacteria in soil**. *PLoS Pathog* 2020, **16**(9):e1008800.

Figures

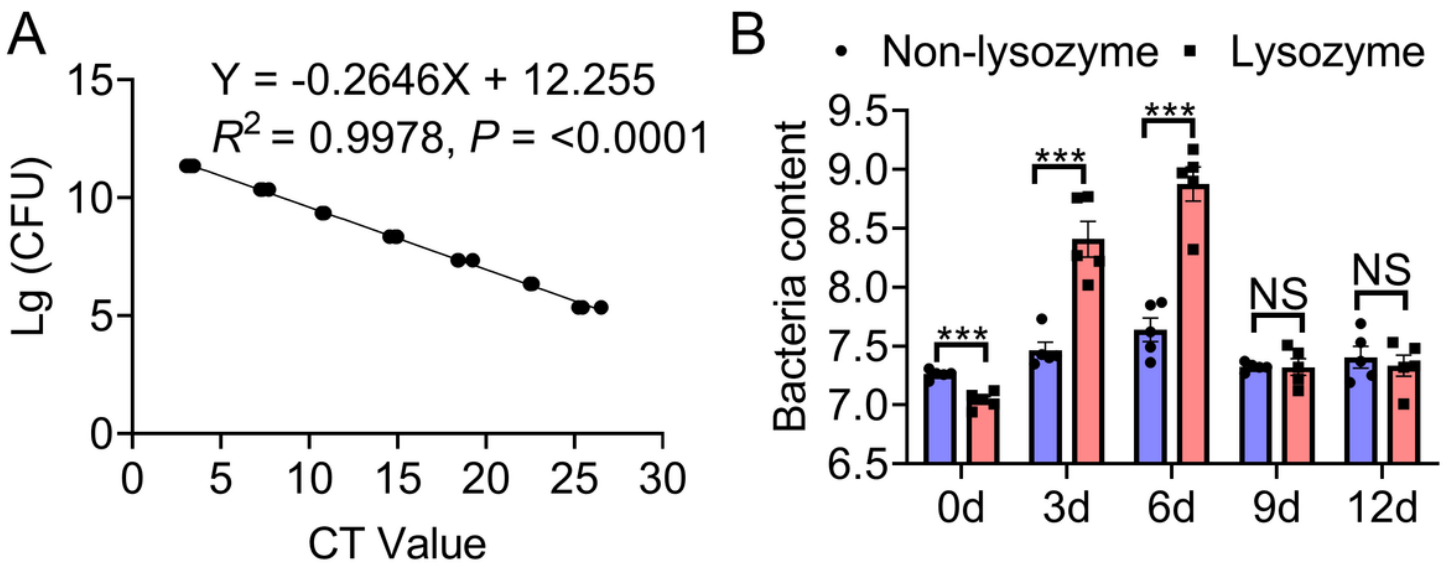

Figure 1

Absolute bacteria content quantified in rectum at different development time. (A) Standard curve generated for bacteria quantification. (B) Absolute bacteria content comparison between lysozyme and non-lysozyme treated samples. Asterisks indicate significant differences (*** P < 0.001, NS: no significance).

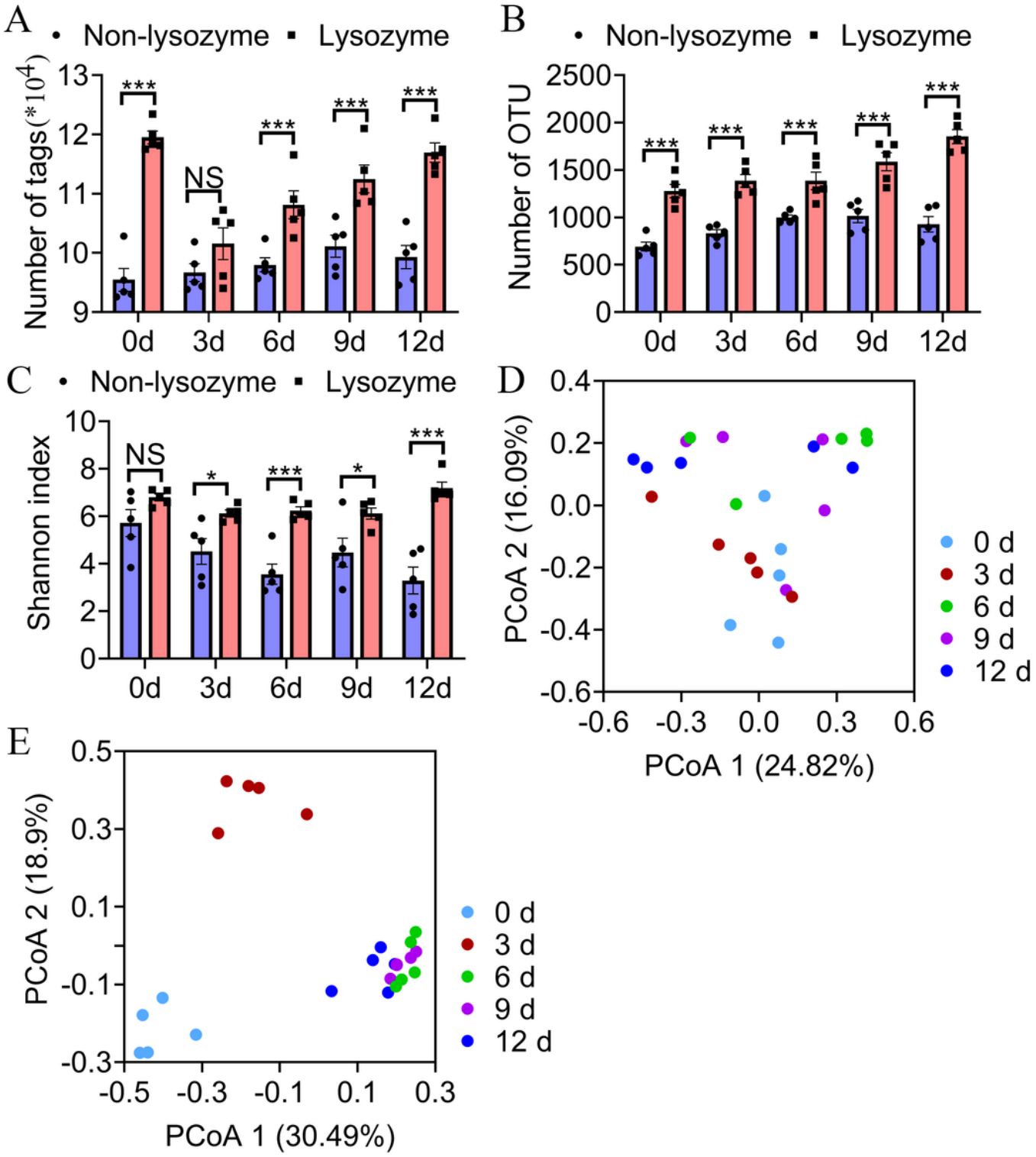

Figure 2

Rectum bacteria diversity investigated by 16S rRNA sequencing. (A), (B) and (C) sequencing tag number, OTU number and Shannon index comparison between lysozyme and non-lysozyme treated samples. Asterisks indicate significant differences (*** $P < 0.001$, NS: no significance). (D) and (E) Bacteria community divergence showed by PCoA for lysozyme and non-lysozyme treated samples.

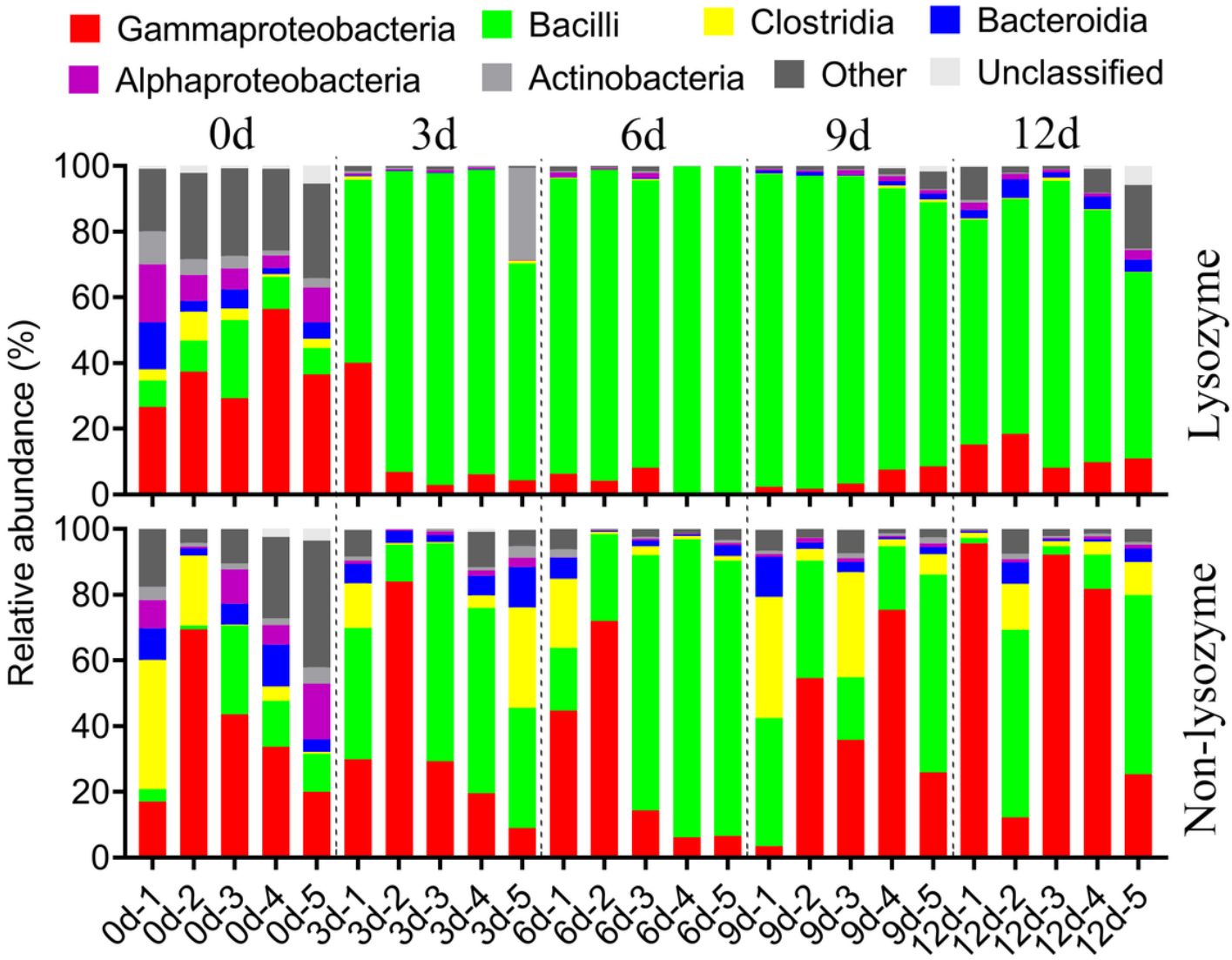

Figure 3

Rectal bacterial composition in lysozyme and non-lysozyme treated rectum at different development time.

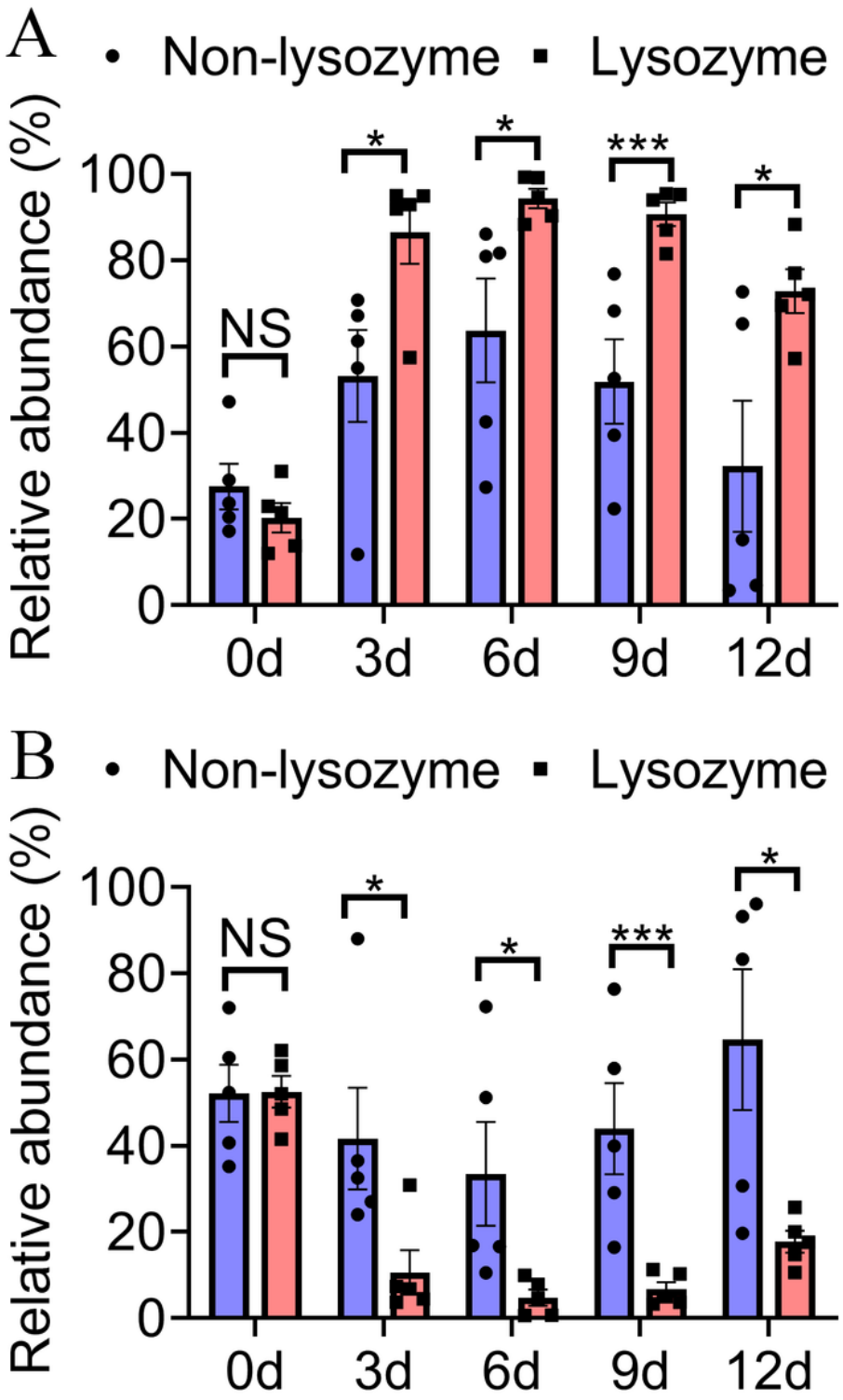

Figure 4

Gram-positive (A) and Gram-negative (B) bacteria relative abundance comparison. Asterisks indicate significant differences (*** P < 0.001, NS: no significance).

Supplementary Files

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- Graphicabstract.tif