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# Characterization of Endophytes Isolated from *Eclipta prostrata* and Roles in Regulating the Gut Microbiota of C57BL/6J Mice

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## Abstract

*Eclipta prostrata* has been extensively studied for its promoting effect on human health. Previous study proved that *E. prostrata* possessed anti-osteoporosis function in SAMP6 mice via gut microbiota (GM). Endophytes are widespread in plants, especially in Chinese herbal medicines. However, little is known regarding the endophytes of *E. prostrata*. In this study, we focus on screening and identifying the endophytes from plant *E. prostrata* and exploring their roles in modulating GM. According to biochemical, physiological tests and 16S rDNA sequence analysis, ten endophytes were characterized in different locations of plant *E. prostrata* belonging to *Lactococcus*, *Bacillus*, *Enterococcus*, *Exiguobacterium* and *Pantoea.* The antimicrobial activity of endophytes EP01-10 was investigated via the oxford cup method. Furthermore, the acid and bile salt resistant ability of EP01-10 was detected to explore their survival ability in gastrointestinal tract. Results indicated that strains of *Lactococcus*, *Bacillus* and *Exiguobacterium* (EP01, 03 and 05) showed strong antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* and strains of *Lactococcus*, *Bacillus* and *Enterococcus* (EP01, 02, 04, 08 and 10) have the ability to survive in the gastrointestinal tract. 16S rRNA sequencing of GM in C57BL/6J mice was performed for diversity and structure analysis responded to the administration of *E. prostrata* extract. *E. prostrata* extract acted on improving the microflora diversity, particularly in increasing the abundance of *Lactococcus* significantly. Thus, as an endophyte, *Lactococcus* plays an important role in *E. prostrata* modulating intestinal tract. Our study suggested that endophytes of Chinese herbal medicines

might be a novel target for the treatment of diseases by regulating the GM.

## Keywords

Endophyte, *Eclipta prostrata*, Gut Microbiota, C57BL/6J Mice, *Lactococcus*

## 1. Introduction

Increasing studies have confirmed that a number of Chinese herbs could regulate the balance of intestinal micro-ecology [1] [2]. Endophytes and their metabolites of Chinese herbs play an important role in the field of agriculture, food, medicine, ecology and have a wide range of antimicrobial activities [3] [4] [5]. The antimicrobial substances of endophytes have low cytotoxicity and are mostly novel [6]. In addition, endophytes can regulate the biosynthesis of plant secondary metabolites [7]. Endophytes including *Bacillus*, *Enterobacter* and *Arthrobacter*, are ubiquitous in plant roots and stems [8]. There were also endophytes from rhizosphere, which is close to common bacteria in soil [9]. Producing antibiotics or plant-promoting biomass plays a significant role in biological control, nitrogen fixation and plant growth [10]. However, the screening of endophytes and how they involve in plants to perform their biological functions in gut remain to be studied.

*Eclipta prostrata*, a traditional Chinese medicine (TCM) of sunflower family, has been used in China for treating uterine bleeding, loose teeth, tinnitus and dizziness [11] [12] [13]. The *E. prostrata* extract (by EtOH) used in this study was qualitatively and quantitatively analysed by UHPLC-Q-TOF-MS and UPLC-MS/MS, respectively [14]. The content of seven main compounds in *E. prostrata* extract was as follows: 0.233 ± 0.005 $mg{\cdot}g^{-1}$ (ecliptasaponin IV), 1.021 ± 0.004 $mg{\cdot}g^{-1}$ (ecliptasaponin A), 0.272 ± 0.003 $mg{\cdot}g^{-1}$ (apigenin), 0.115 ± 0.003 $mg{\cdot}g^{-1}$ (3'-hydroxybiochanin A), 0.306 ± 0.004 $mg{\cdot}g^{-1}$ (luteolin), 0.125 ± 0.008 $mg{\cdot}g^{-1}$ (luteolin-7-O-glucoside), 0.271 ± 0.001 $mg{\cdot}g^{-1}$ (wedelolactone). Pharmacological studies have shown that *E. prostrata* extract displays anti-osteoporosis effects by modulating gut microbiota (GM) [15]. *E. prostrata* contains such various active ingredients widely distributing in plants and might contribute to a good environment for endophytes.

In this study, various endophytes were isolated from wild plant samples of *E. prostrata*, some of which had significant inhibitory effects on *Staphytococcus aureus* and *Escherichia coli*. However, the relationship of endophytes of *E. prostrata* and GM is unclear. Based on the survival ability in gastrointestinal tract, endophytes of *E. prostrata* were further analysed. 16S rRNA sequencing was conducted to assess the effect of *E. prostrata* on the GM community and endophytes involved in regulating the GM of mice would be figured out. Screening and charactering endophytes from TCM is a hot field, which expand the study of pharmacodynamics and underlying mechanism via GM.

## 2. Methods and materials

### 2.1. Microorganisms and Culture Conditions

The microorganism EP01-10 listed in Table 1 used in this study isolated from *E. prostrata* herbs sample including six *Bacillus* strains, one *Lactococcus* strain, one *Exiguobacterium* strain, one *Pantoea* strain, one *Bacterium* strain and the indicator strains of *E. coli* CMCC44103 and *S. aureus* ATCC6358 were preserved at microbiology laboratory of Tianjin University of traditional Chinese medicine. All the medium components used in this study were reagent pure grade purchased from Solarbio Co., Ltd. (Beijing, China). All other chemicals used were of analytical grade unless otherwise stated. Solid media used in the antibacterial study consisted of MRS agar (potato, 200 g; glucose, 20 g; agar, 18 g; and distilled water, 1 L) and Luria-Bertani agar (pep-tone, 10 g; yeast extracts, 5 g; NaCl, 10 g; agar, 18 g; and distilled water, 1 L)., the same agar-lacking liquid media, were used for fermentation test. The strain EP01-10 was activated by transferring single colonies of the strain from plates to 10 mL activation LB or MRS medium. The flasks were shaken at 37˚C, 150 rpm for 16 h.

### 2.2. Experimental Animals

Experimental animals including 2-month-old female healthy mice C57BL/6J (SPF level) were provided by Huafukang Co., Ltd. (Beijing, China). Animals were raised in institute of radiation medicine Chinese academy of medical sciences (Tianjin, China), at room temperature (22˚C ± 2˚C), with relative humidity of 58% - 65% and light cycle of 12 h alternation. In this study, 10 same-treated mice were tested for each group (5 per cage).

### 2.3. Preparation of Raw Material and dose of *E. prostrata*

*E. prostrata* were collected in April 2018 from the Jiulongshan of Qingyuan

Table 1. Colony characteristics of endophytic strains in different location of *E. prostrata.*

| Name | Location in *E. prostrata* | Colony Characteristics | The highest homology strain |
|---|---|---|---|
| EP01 | Root | medium size, raised, slightly white, moist, with neat edges | *Lactococcus* sp. ARa2 |
| EP02 | Leaf | white, round, smooth and opaque, with neat edges | *Enterococcus faecium* |
| EP03 | Stem | white, round, smooth and opaque, with neat edges | *Bacillus megaterium* |
| EP04 | Root | white, round, smooth and opaque, with neat edges | *Bacillus* sp. WS22 |
| EP05 | Leaf | yellow, smooth, edge regular, protruding | *Exiguobacterium acetylicum* |
| EP06 | Root | white, round, smooth and opaque, with neat edges | bacterium L35 |
| EP07 | Stem | round, yellow, edge neat, low convex, smooth | *Pantoea agglomerans* |
| EP08 | Leaf | white, round, smooth and opaque, with neat edges | *Bacillus megaterium* |
| EP09 | Root | white, round, smooth and opaque, with neat edges | *Bacillus aryabhattai* |
| EP10 | Root | colorless, transparent or translucent, relatively flat, with neat and smooth edges | *Bacillus cereus* |

(Guangdong, China). The surface of fresh *E. prostrata* was cleaned with sterile water and dried at room temperature. Under sterile conditions, 100 mg of roots, stems and leaves were cut into tubes, respectively. 1 mL of sterile miliQ water was added, and samples were centrifuged at 4000 rpm for 1 min. The supernatant was collected for the following endophytes screening.

According to previous study [15], 2 kg of dried *E. prostrata* was refluxed by 70% EtOH for twice (2 h per time), then the extract was concentrated under vacuum to 200 g and stored at 4˚C for feeding. The group raw dose of *E. prostrata* (EP) for treating P6 mice was designed as 4.8 g·kg$^{-1}$, correspondingly the extract dose was 0.48 g·kg$^{-1}$ (**Table 2**). The above extracts were dissolved into water for regularly feeding per mice per day for 4 weeks (EP, n = 10) and another ten mice were chow fed as control group (control, n = 10). All mice in this study meet the SPF level nutrition needs.

## 2.4. Screening and Identification of Endophytes of *E. prostrata*

Each 50 μL of supernatant were coated on LB and MRS plates and cultured at 37˚C for 24 h. By observing the colony characteristics on the plates, different phenotypes of bacterial colonies were selected from the plate for identification. The genomic DNA of strains was extracted by kit (Tiangen, China) and used as PCR templates. The 16S rDNA sequence was amplified and sequenced using universal primers 27F: 5'-AGAGTTTGATCATGGCTCAG-3' and 1492R: 5'-ACGGTTACCTTGTTACGACTT-3'. The amplification was conducted by polymerase chain reaction in a PCR thermal cycler (Bio-Rad, USA). The PCR amplification system and reaction factor was according to previous work [16]. The amplified products were detected by agarose gel electrophoresis and then purified by kit (Tiangen, China). The 16S rDNA sequence of each isolate was then sequenced by BGI Biotech Co., Ltd. (Beijing, China). According to sequencing results, the closest relative was searched in GenBank database using NCBI-nBLAST.

## 2.5. Antimicrobial Activity and Survival Ability in Gastrointestinal Tract Tests of Endophytes of *E. prostrata*

*E. coli* and *S. aureus* were used as the indicator strains for the following assay to detect the antimicrobial activity of the screening strains according to Zhao *et al.* [16]. The sterilized water and 0.4 mg/mL gentamicin were used as negative control and positive control, respectively. Plates were cultivated at 37˚C for 24 h, and antimicrobial circle diameter was measured. Each experiment was repeated 3 times.

**Table 2.** Dose of *E. prostrata* for each group.

| Group | Mice (feeding) | Raw dose | Extract dose |
|---|---|---|---|
| control | C57BL/6J (chow-fed) | - | - |
| Pre-EP | C57BL/6J (chow-fed) | - | - |
| Post-EP | C57BL/6J (chow + *E. prostrata*-fed) | 4.8 g·kg$^{-1}$ | 0.48 g·kg$^{-1}$ |

Survival ability in gastrointestinal tract tests included acidic tolerance and bile salt tolerance experiments. MRS medium with pH values of 3.0, 4.0, 5.0 and 7.0 (control) was prepared with 37% hydrochloric acid (based on the measured values after autoclave) were used for acidic tolerance test. 0.5%, 1% and 2% of bile salt was added into MRS medium for bile salt tolerance test, and the medium without bile salt was used as control. Acidic tolerance and bile salt tolerance tests were evaluated by plating and colony counting after 3 h of cultivation. The survival rate was calculated by the colony number of experimental group divided that of control group.

### 2.6. Fecal Sample Collection and Illumina Sequencing

Fresh stool samples of each group (3 of 10 mice per group) were collected in sterile tubes, then frozen and stored at −80˚C. Afterwards, total genome DNA was extracted by kit (Tiangen, China) from samples and monitored by agarose gels. Afterwards, DNA samples were diluted to 1 $ng{\cdot}\mu L^{-1}$ using sterile water and then sent for sequencing (Novogene, Beijing, China). The bacterial 16S rRNA gene V4 region was amplified by PCR with primers using 515F 5'-GTGCCAAGCMG CCGCGGTAA-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3'. All PCR reactions were carried out and PCR products were conducted according to Zhao *et al.* [15], the library was sequenced on an Illumina HiSeq 2500 platform and average 250 bp paired-end reads were generated.

### 2.7. Bioinformatics Analysis

Raw tags were performed to QIIME quality controlled process (V1.7.0) under specific filtering conditions to obtain the high-quality clean tags (effective tags) [17]. Operational taxonomic units (OTUs) were clustered with ≥97% sequence similarity by Uparse software (Uparse v7.0.1001, USA) [18]. Representative sequence for each OTU was screened in Green Gene Database for taxonomic annotation [19]. Different taxonomic levels classified from OTUs were conducted using the MUSCLE software (V3.8.31, USA) [20]. Subsequent analysis of alpha diversity (including observed-species and chao1 index) and beta diversity (including Principal coordinate analysis (PCoA)) were all performed basing on this output normalized data and further statistically calculated with QIIME and displayed with R software (V2.15.3). The enriched and significant bacteria in each group were identified by linear discriminant analysis (LDA) combined with effect-size measurements (LEfSe), with $P < 0.05$ [15].

## 3. Results

### 3.1. Morphology Characteristics of Endophytes in Different Location of Plant *E. prostrata*

Five strains of bacteria were screened from the roots, two from the stems and three from the leaves of *E. prostrata* (**Table 1**). Among them, the bacterial colonies screened from roots and leaves had common characteristics of white, round,

smooth and opaque surface, neat edges and Gram staining positive. By morphological analysis, these bacteria are similar to the most common rhizosphere bacteria, *Bacillus* spp. In addition, there was colony of EP07 screened from stems with round, yellow, edge neat, low convex, smooth and Gram negative characteristics, which has been rarely reported.

### 3.2. 16S Ribosomal DNA Sequence Analysis

The 16S rDNA sequence of the amplified PCR product was determined (Figure 1). Sequences of 1500 bp fragments (supplementary data S1) showed similarity (minimum identity 98%) with the responding strains blasting in Genbank, and the highest homology strains were showed in Table 1. Both molecular and phenotypic characterization showed that the all the ten strains screened from *E. prostrata* might belong to *Bacillus* sp., *Lactococcus* sp., *Enterococcus* sp., *Exiguobacterium* sp., and *Pantoea* sp..

### 3.3. Bacteriostatic Activity of Endophytes of *E. prostrata*

By oxford cup method, the bacteriostasis activity of 10 strains of bacteria isolated and identified was tested. Strain EP01, 03, 05 showed strong antimicrobial activity against *E. coli* and *S. aureus* (shown in Figure 2). Strain EP02, 04, 06, 07, 08, 09 and 10 have no bacteriostasis to these two pathogenic bacteria. Inhibitory zone of strain with antimicrobial activity isolated from *E. prostrata* was shown in Table 3. Combining with previous studies, strains with inhibitory activity are widely distributed in roots, stems and leaves. The bacteriostatic activity of strain EP01 is stronger than that of others. However, strain EP08 and EP09 were also *Bacillus*, which have no inhibitory activity. The antibiotic gentamicin in positive control group was more sensitive to *E. coli* and *S. aureus*.

### 3.4. Survival Potential of Endophytes in Gastrointestinal Tract

Based on the gastrointestinal environment, medium with high concentration of

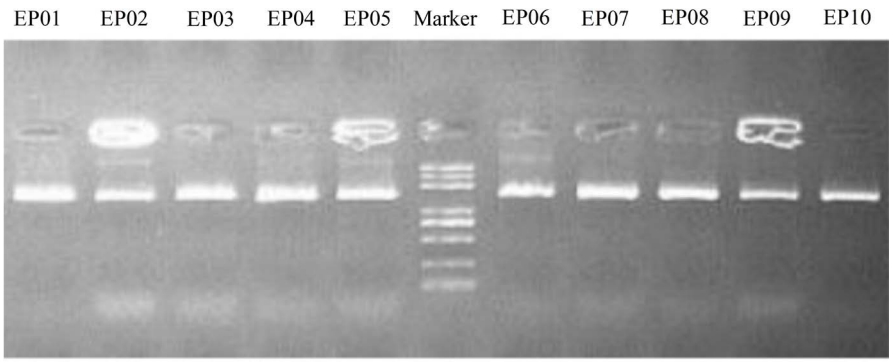


Figure 1. Agarose gel electrophoresis of aimed DNA detected from strain EP01-10. Electrophoresis conditions: 3 ul sample + 1% agarose gel, Marker band composition: 100 bp, 250 bp, 500 bp, 750 bp, 1000 bp, 2000 bp, 3000 bp, 5000 bp. The concentration of 750 bp bands was 60 ng/3uL, which showed a bright band, and the other bands were 30 ng/3uL. The direction of electrophoresis is from top to bottom.

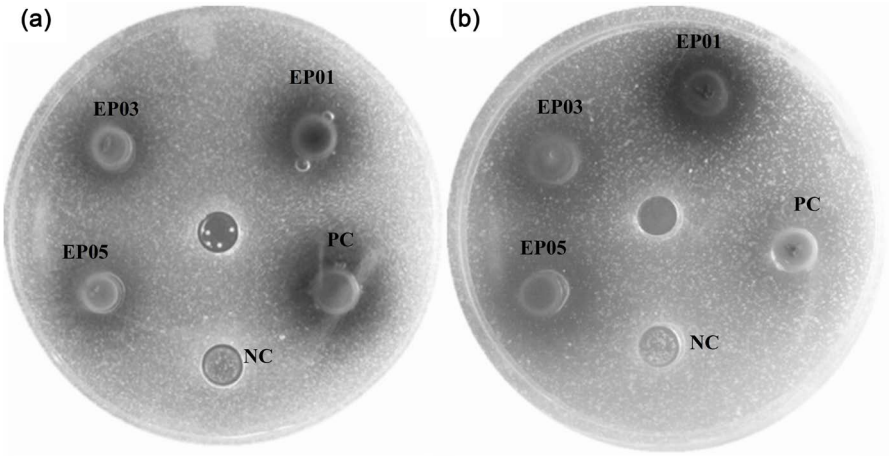


**Figure 2.** Antimicrobial activity of strains isolated from *E. prostrata*. Indicator strains were *E. coli* (a) and *S. aureus* (b). NC, negative control miliQ water; PC, 0.4 mg/mL gentamicin.

**Table 3.** Inhibitory zone of strains isolated from *E. prostrata*.

| Indicator | Inhibition zone (mm) | | | |
|---|---|---|---|---|
| | EP01 | EP03 | EP05 | PC |
| *S. aureus* ATCC6358 | 16.33 ± 0.21 | 16.77 ± 0.36 | 17.66 ± 0.12 | 12.43 ± 0.04 |
| *E. coli* CMCC44103 | 16.32 ± 0.45 | 13.51 ± 0.01 | 14.38 ± 0.21 | 23.21 ± 0.09 |

acid and bile salt were used for survival test. Considering that survival rate of bacteria under the condition of pH 7.0 and without bile salt was 100% (control), survival rate under other condition was calculated as colony numbers divided by control (Figure 3). Under pH 3.0, the survival rate of strain EP01, 02, 04, 08 and 10 was decreased to >60% after 3 h of cultivation, while the survival rate was >80% under pH 4. After growing with 0.5%, 1% and 2% bile salts for 3 h, the number of EP01, 02, 03, 04, 08, 09 and 10 did not decrease compared with the number without bile salts. All in all, endophytes including EP01, 02, 04, 08 and 10 have the ability to survive in the gastrointestinal tract.

### 3.5. Bacterial Community of Gut Microbiota Altered by *E. prostrata*

By 16S rRNA sequencing, an average of 732 OTU clusters were obtained. We found that the GM diversity of Post-EP mice was higher than that of Pre-EP mice by calculating observed species and Chao 1 of alpha diversity index (Figure 4(a)). Unweighted uniFrac-based PCoA of beta diversity revealed a distinct clustering of microbiota composition for each group (Figure 4(b)). Diversity analysis indicated remarkable differences of GM between Pre-EP and Post-EP. At the phylum level, Firmicutes, Bacteroidetes and Proteobacteria constituted the three dominant phyla in all samples (Figure 4(c)). In Post-EP group, the abundance of Firmicutes was significantly ($p < 0.01$) more than that of Pre-EP (Figure 4(d)).

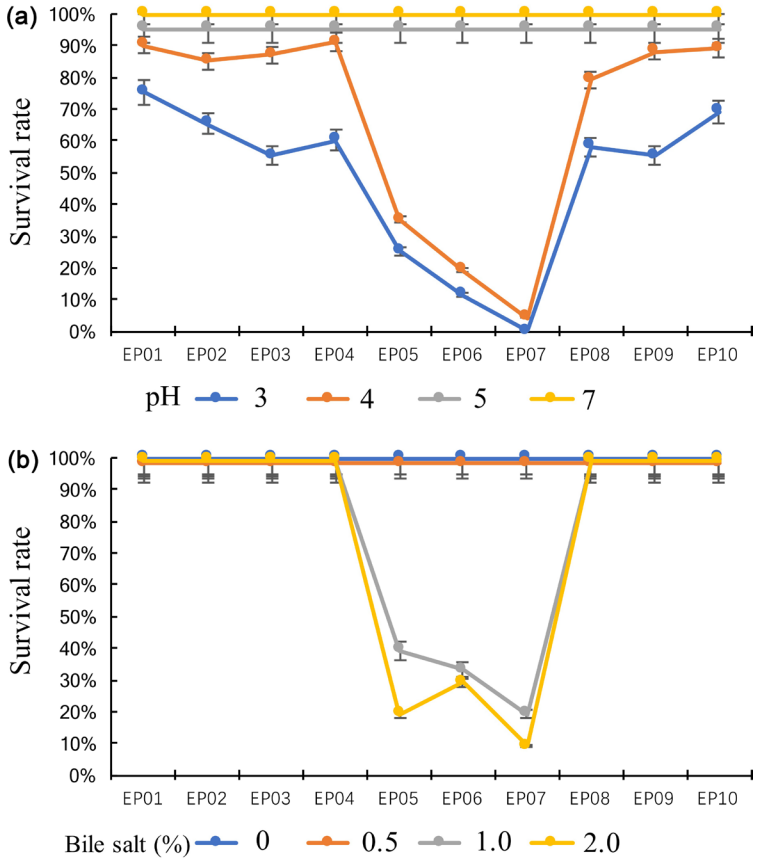


**Figure 3.** (a) Acid tolerance and (b) bile salt tolerance tests of endophytes of *E. prostrata.*

### 3.6. *E. prostrata* Modulates Gut Microbiota by Targeting *Lactococcus* Identified as an Endophyte

Heat map showed top 20 genus levels abundant in this study (**Figure 5(a)**). In the GM of EP-feeding mice (Post-EP), bacterial genera including *Odoribacter*, *Atopobium*, *Prevotellaceae* UCG-004, *Lactococcus* and *Lactobacillus* were more than the proportion of Pre-EP group; and bacterial genera including *Prevotellaceae* UCG-001, *Bacteriodes*, *Blautia*, *Clostridium*, *Allobaculum* and *Faecalibaculum* were less than the proportion of Pre-EP group. We further applied LDA combined LEfSe to explore the biomarkers in each group and relative richness of the bacterial community at various levels. **Figure 5(b)** summarizes the enrichment and variations in bacterial community in each group. At the genus level, *Bacteriodes*, *Clostridium*, *Allobaculum* and *Faecalibaculum* were enriched in group before treatment; *Lactobacillus*, *Atopobium*, *Prevotellaceae* UCG-004, *Lactococcus* and *Solobacterium* were enriched in the mice group treated with *E. prostrata*. Compared with Pre-EP group, the abundance of genus *Lactococcus* was higher than that of Post-EP group. Among *Lactococcus*, the abundance of species including *L. garvieae*, *L. raffinolactis* and other species was remarkably increased after *E. prostrata* treatment. However, *Lactococcus lactis* showed the opposite. Thus, as an endophyte, *Lactococcus* was an important target of the plant in intestinal tract (**Figure 5(c)**).

## 4. Discussion

As a TCM, *E. prostrata* has a significant regulatory effect on GM. Recent study has shown that *E. prostrata* could improve the mice bone health by altering GM [15]. The bacterial diversity was enriched and the microflora structure were

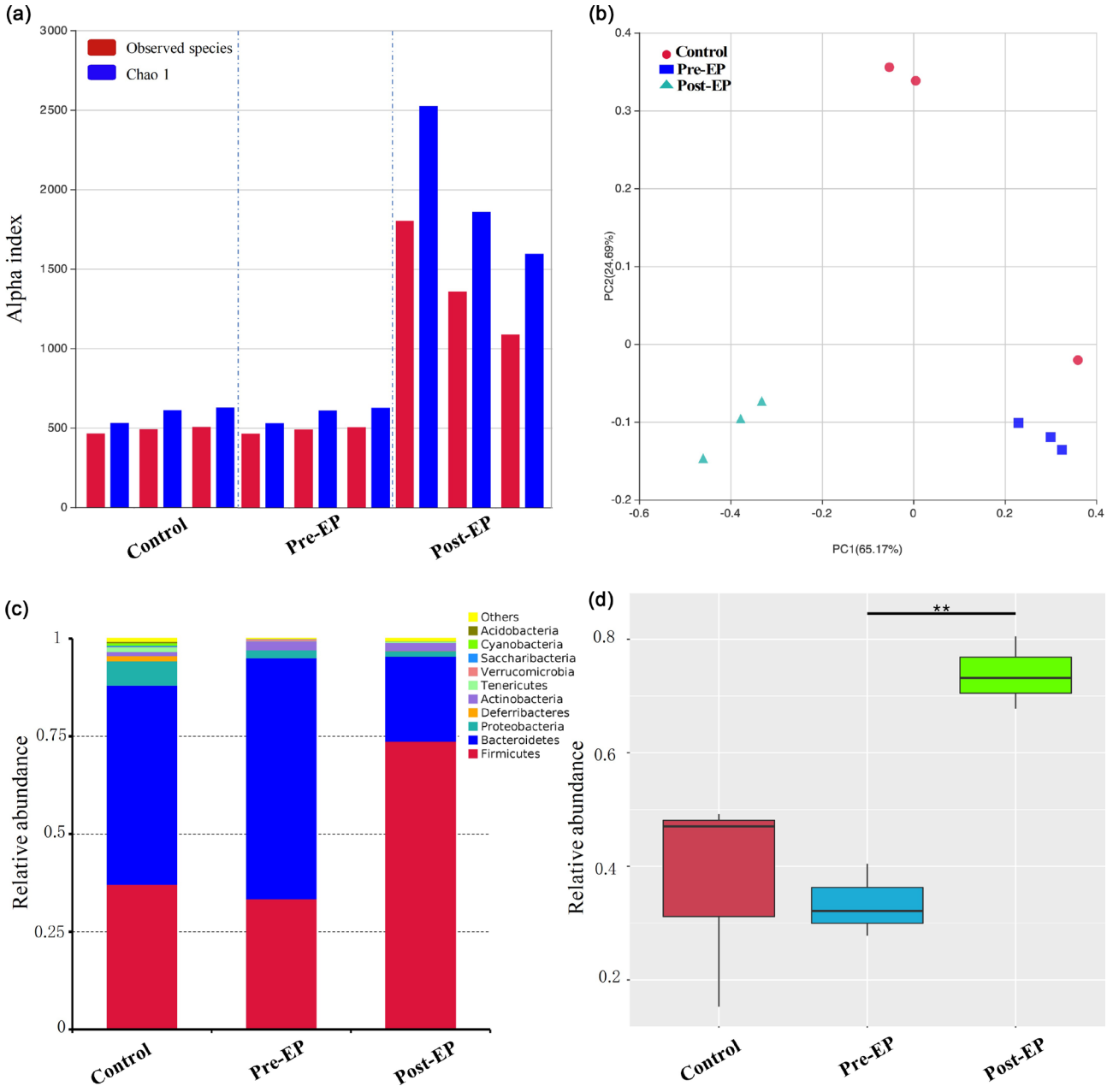


**Figure 4.** A diversity analysis of microbiota composition for each treatment group. Alpha diversity analysis of (a) Observed species and Chao1; (b) Weighted UniFrac-based principal coordinates analysis (PCoA). The x-axis represents a principal component, and the y-axis represents another principal component, and the percentage represents the contribution of the principal component to the sample difference; each point in the fig. represents a sample, and the same group of samples is represented by the same color; (c) The relative bacterial abundance of each group at the phylum level; (d) MetaStat statistical analysis of phylum significance difference between groups. The horizontal axis is the sample grouping; the vertical axis is the relative abundance of corresponding species. "*" means the difference between the two groups is significant ($P < 0.05$), "**" means the difference between the two groups is very significant ($P < 0.01$).

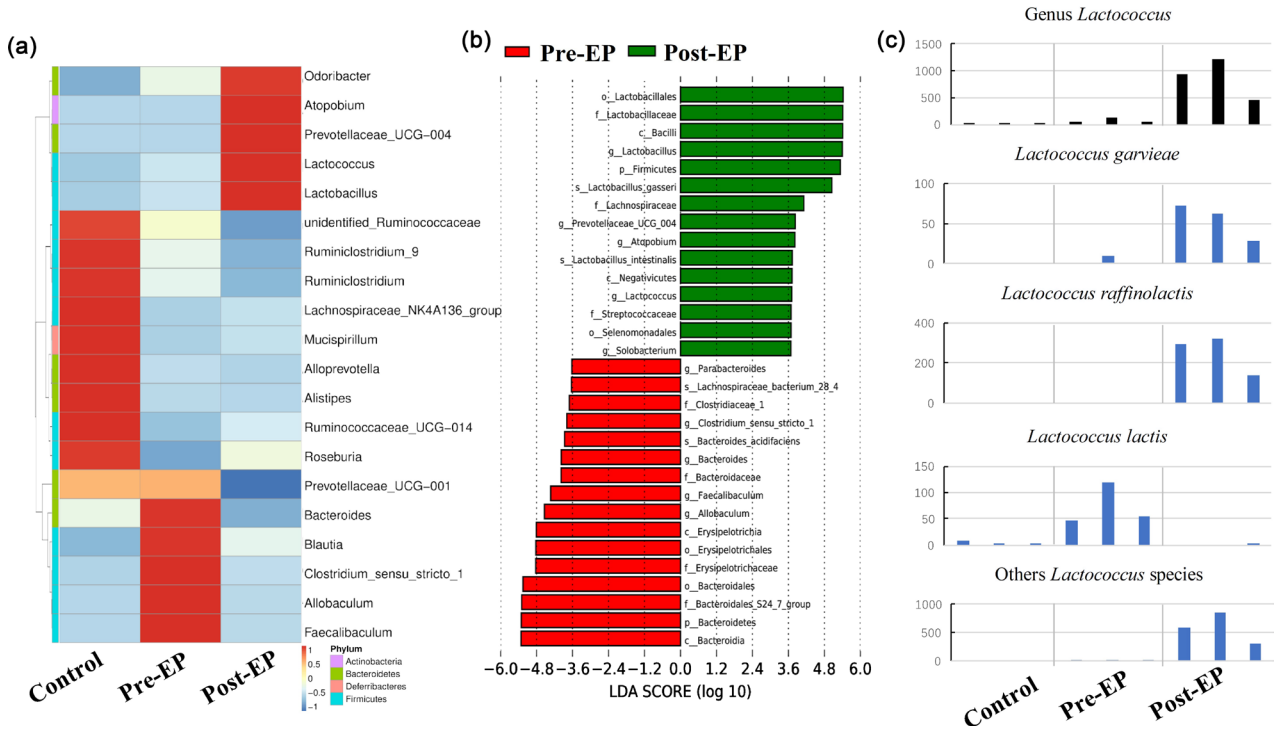


**Figure 5.** (a) The relative abundance of bacterial communities at genus level; (b) LEfSe analysis result of Post- and Pre-EP group. The species with different abundance are shown. The length of the histogram represents the size of the different species (*i.e.* LDA score); (c) The absolute abundance of *Lactococcus* genus and species in each group.

altered after *E. prostrata* treatment. *E. prostrata* had the effect of improving the phylum Firmicutes and genus *Lactobacillus*, *Lactococcus* and *Atopobium* of different animal models, indicating that they might be the potential target of *E. prostrata* via GM. Moreover, *Lactococcus* strain was isolated from plant *E. prostrata* and had the ability to survive in the gut. However, some endophytes of *E. prostrata* cannot be isolated in lab, or detected by 16S sequencing, which limits the study of endophytes of *E. prostrata* efficacy on GM.

Compared with endophytic fungi in TCM, studies on endophytic bacteria in TCM are fewer. Metabolites produced by endophytes, especially antimicrobial substances, can inhibit the growth of pathogens and regulate intestinal disorder [21] [22]. Antimicrobials were reported to contribute to microbiota-induced susceptibility to obesity and metabolic diseases [23]. Gastrointestinal infections are a major concern in human health, but antibiotics cause a harmful effect on GM [24]. Therefore, the anti-infective effect of endophyte-producing antimicrobials is a promising alternative to antibiotics, especially for particular cases where other methods are not allowed (e.g., pregnant women) [25]. *Bacillus*, *Lactococcus* and *Exiguobacterium* isolated in this study are major producers of antimicrobial substances. Lactic acid bacteria (LAB) constitute a group of microorganisms that can produce bacteriocins, which are proteinaceous antimicrobial molecules. According to Alvarez-Sieiro *et al* [26], putative bacteriocin gene clusters of three lanthipeptides, seven sactipeptides, one LAP, twenty class ii and one class iii were identified in thirteen genomes of *Lactococcus* strains. In

addition, two class iii putative bacteriocin gene clusters were identified in four genomes of *Exiguobacterium* strains. Gene clusters of known and putative bacteriocins, non-ribosomally synthesized peptides (NRPs), polyketides (PKs) and other antimicrobials were also widely distributed in various *Bacillus* genomes [27].

Probiotics represent one of the fastest growing consumer items on the functional food because they are benefiting human health via GM [28]. Endophytes isolated from edible Chinese herbal medicine can colonized in gut means that it has the potential to develop into probiotics [29]. Therefore, the research and application of endophytic bacteria in traditional Chinese medical plants have broad prospects. Moreover, the interaction of endophytic bacteria and Chinese herbal medicines remains to be studied.

In recent years, the ecological environment has been severely damaged, the predatory utilization of medicinal plants and the difficulty of introduction and cultivation of many wild precious medicinal plants have resulted in serious shortage of natural medicinal resources and lower content of active ingredients. One of the possible reasons is the lack of endophytes, which contribute to form such abundant secondary metabolites for human needs [30]. Although research on endophytes has already attracted people's attention for 20 years, the application of endophytic bacteria in TCM has not drawn too much attention [31] [32]. The combination of the research of endophyte and TCM, especially in resolving resource problems, and has great economic and theoretical value.

## 5. Conclusion

In conclusion, *E. prostrata* plant is rich in endophytes mainly including *Lactococcus* and *Bacillus*, which showed strong antimicrobial activity against *E. coli* and *S. aureus*, and have the ability to survive in the gastrointestinal tract. *E. prostrata* extract ameliorated the microflora diversity, particularly in increasing the abundance of *Lactococcus* significantly, indicating that endophyte *Lactococcus* contributed to *E. prostrata* modulating GM.

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## Ethical Statement

This study was carried out in strict accordance with the recommendations in the

Guidance Suggestions for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of China. The protocols were approved by the Laboratory Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (Permit Number: TCM-LAEC20180028).

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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## Supplementary Data S1

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