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# *Lactobacillus casei* Zhang exerts probiotic effects to antibiotic-treated rats

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ABSTRACT

Probiotics administration can facilitate the restoration of host gut microbiota/metabolome after antibiotic treatment. Yet, the mechanism behind such beneficial effects remains unclear. This study constructed a rat model of antibiotic-induced gut dysbiosis to monitor the effects and mechanism of probiotic (*Lactobacillus casei* Zhang) treatment in maintaining gut homeostasis and restoring the gut microbiota/metabolome. Forty rats were randomly divided into four groups (n = 10 per group): control receiving only saline (Ctrl), antibiotic (AB-Ctrl), antibiotic followed by probiotic (AB-Prob), and antibiotic plus probiotic followed by probiotic (AB + Prob). Rat fecal microbiota and sera were collected at four time points from pre-treatment to post-treatment. The probiotic-treated group (AB + Prob) had significantly more *Parabacteroides* (*P.*) *goldsteinii* after one week of antibiotic and probiotic intervention but fewer antibiotic resistance genes (ARGs)-possessing bacteria (*Clostridioides difficile* and *Burkholderiales* bacterium). Consistently, metabolomics data revealed that both probiotic groups had more acetic acid, propionic acid, butyric acid, and valeric acid post treatment. Moreover, a potential probiotic species, *P. goldsteinii*, strongly correlated with *L. casei*, as well as propionic acid, butyric acid, and valeric acid. Furthermore, administering probiotic lowered the serum IL-1α level. In contrast, the antibiotic-recipients had a higher irreversible level of IL-1α, suggesting inflammation of the rats. Thus, antibiotic treatment not only led to host gut dysbiosis, but inflammatory responses and an increase in gut ARGs. Daily *L. casei* Zhang supplementation could alleviate the side effect of cefdinir intervention and facilitate the restoration of gut microbial homeostasis, and these probiotic effects might involve *P. goldsteinii*-mediated beneficial activities.



## 1. Introduction

The gastrointestinal tract is colonized by trillions of microbes that maintain a healthy symbiotic relationship with their host. Therapeutic intervention with antibiotics has a history of almost 100 years, and it has contributed significantly to raise the survival rate of previously intractable microbial infections [1]. Cefdinir is a third-generation oral cephalosporin antibiotics against both Gram-positive and Gram-negative bacteria. It has been widely used to treat certain bacterial infections e.g. bronchitis, pneumonia, infections of the ears, sinuses, and throat [2]. However, the overuse of antibiotics can lead to serious side effects and increase in risk of spreading antibiotics resistance. Moreover, since antibiotics does not distinguish between normal microbiota and harmful bacteria, antibiotic therapies can disturb the gut microbiota community, thus resulting in gut dysbiosis. Gut dysbiosis is associated with a wide spectrum of diseases, such as irritable bowel syndrome, inflammatory bowel disease and obesity [3–5]. Commonly, this was ascribed to the perturbations of the gut environment, microbiota, and metabolome [4].

Probiotics are live microorganisms that confer health benefits on the hosts when administered in adequate amounts. Actually, they can prevent and relieve symptoms of many gastrointestinal diseases through various mechanisms, e.g., by competing with pathogenic microbes for available nutrients and epithelial binding sites, strengthening the gut barrier structure and function, enhancing host immunity, and modulating the stability, expression, and composition of host-microbiota either directly or indirectly [6].

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Several studies have demonstrated the efficacy of *Lactobacillus* in alleviating gut-related disorders or metabolic diseases [7,8]. The relief of clinical symptoms is often reported to be at least partially accompanied by gut microbiota modulation [9]. However, some strains did not show any positive effects in clinical scenarios, which was reflected by the failure in recovering changes in the gut microbiota and inflammation caused by antibiotics [10]. Thus, the probiotic effects are strain-/individual-specific and need to be systematically determined.

The probiotic effects of the koumiss-originated strain, *Lactobacillus casei* Zhang, have been widely investigated by various *in vivo* studies at the population level [11,12]. This study aimed to explore the effects and the protective mechanism of *L. casei* Zhang in maintaining and restoring a homeostatic gut microbiome when encountering drastic external challenges like antibiotic application using a rat model. Given the common medical use of antibiotics and its severe perturbation of the gut microbial balance, the effects of administering *L. casei* Zhang on restoring microbiota and protecting from excessive inflammation in rats treated with antibiotics were investigated. Cefdinir was given to rats for one week to induce gut dysbiosis, followed by a two-week recovery with or without giving *L. casei* Zhang. Changes in the gut microbiota and metabolites were then compared between different treatment groups using combined omics approach. The findings of this work provide important practical implications for understanding the role and mechanism of probiotics in protecting from gut dysbiosis caused by antibiotics. Moreover, the obtained data serve as evidence supporting the safe use of probiotics in clinical settings when antibiotics are concurrently applied.

## 2. Material and methods

### 2.1. Animals and ethics statement

Forty specific-pathogen-free male Sprague-Dawley rats (190–200 g in weight) were used (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China). The rats were housed in wire-mesh cages in an air-conditioned room (22 °C ± 1 °C) maintained at a constant relative humidity (50%−60%) under 12-h light–dark cycle. Rats received free access to drinking water (replacement at 3-day intervals) and food (commercial standard rodent chow: 18.0% protein, 4.0% lipids, and 5.0% fiber) during experiments. The animal procedures were carried out by the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee on Animal Research of Inner Mongolia Agricultural University ([2020]044).

### 2.2. Experimental design

The experimental design is illustrated in Fig. S1. Forty rats were acclimatized for one week before the start of the experiment. They were then randomly divided into four groups (n = 10 rats per group): control (Ctrl), antibiotic (AB-Ctrl), antibiotic followed by probiotic (AB-Prob), and antibiotic plus probiotic followed by probiotic (AB + Prob). The application of cefdinir for one week was meant to induce dysbiosis in the rats, and the two-week probiotic treatment was to test the effects of probiotic on gut dysbiosis recovery. Cefdinir (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate-buffered saline, and the antibiotic dosage (135 mg/kg/d per rat by oral gavage twice daily) was applied [13]. The probiotic strain, *L. casei* Zhang, was provided by the Lactic Acid Bacteria Collection Center, Inner Mongolia Agricultural University, China. It was prepared and given by oral gavage ($2.5 \times 10^9$ CFU/day [14] in a sterilized saline solution twice daily). For the AB-Ctrl group, cefdinir was administrated for 1 week, followed by saline treatment for 2 weeks. The AB-Prob group was given the probiotic instead of the saline given to the AB-Ctrl group. For the AB + Prob group, probiotic was given three hours after cefdinir administration for 1 week, followed by probiotic treatment for 2 weeks. The control group was given an equivalent volume of sterilized 0.85% saline solution [15,16] instead of antibiotic or probiotic, respectively. Blood of tail-vein and fecal samples were collected before the first administration (Day 0), and at Days 7, 14 and 21, respectively. Changes in the gut microbiota, SCFAs, and serum immune factors were compared between different treatment groups at different time points. A total of 160 shotgun metagenomic samples with 160 matched metabolic samples were analyzed to investigate the effects of *L. casei* Zhang on the recovery of the intestinal microbiota/metagenome.

### 2.3. Enumeration of serum inflammatory cytokines

Blood samples (0.8–1 mL) were collected in heparin-containing tubes, followed by centrifugation at 3000 g at 4 °C for 10 min. The resulting supernatants were stored at −80 °C. The levels of multiple cytokines, including granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-2, IL-12p70, granulocyte–macrophage (GM-CSF), IL-4, IL-13, interferon (IFN)-γ, IL-5, IL-17A, IL-1α, IL-6, tumor necrosis factor (TNF)-α, IL-1β, IL-10, eotaxin, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-2, growth regulated oncogene-α (GROα), MCP-3, regulated upon activation normal T cell expressed and secreted (RANTES), IFN-γ-induced protein (IP)-10, and MIP-1α, were determined using ProcartaPlexv multiplex immunoassay (Thermo fisher Co. Ltd., China). Moreover, the levels of IL-8, lipopolysaccharide (LPS), diamine oxidase (DAO), C-reactive protein (CRP), complement3 (C3), IgG, and D-lactate (D-LA) were determined using respective ELISA kits following the manufacturer's protocols (Quanzhou Ruixin Biological Technology Co. Ltd., China).

### 2.4. Fecal sample collection, DNA extraction, and metagenome sequencing of fecal microbiota

Fresh fecal samples were collected in 5 mL sterile Eppendorf tubes and immediately stored at −80 °C until further analysis. Fecal genomic DNA was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) in a cleanroom. The extracted DNA was checked for quality and quantified using 1% agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometer (optical density, OD, ratio of OD260/280 was determined). For metagenome sequencing, ~5 μg of DNA per sample was used for constructing shotgun library and subsequent sequencing was performed on an Illumina Hiseq 2500 platform by Novogene Co. Ltd., Beijing, China. Approximately 6 Gb of metagenomics data was generated for each sample.

### 2.5. Sequence analysis

Whole-genome shotgun sequencing allows relative quantification of the gut microbiota and enables gene and functional profiling. Raw reads were quality controlled using KneadData (version 0.6.1), which integrated several QC tools such as FastQC and Trimmomatic [17]. After trimming low-quality reads and dropping reads <100 bp in length, bowtie2 was used to map the filtered reads to the human genome (hg19) to remove host contaminants [18]. The remaining high-quality reads were loaded into the MetaPhlAn2 (version 2.2.0) pipeline for taxonomy profiling with default parameters [19].

### 2.6. GC–MS analysis

Fecal samples (150 mg/sample) were thawed, before mixing with 1000 μL of aqueous NaOH containing IS (10 μg $mL^{-1}$ caproic acid-d3) and homogenizing for 10 min. Samples were then centrifuged at 14,000 g at 4 °C for 20 min. Supernatants were then collected and re-dissolved in 300 μL of water. Standard solution was prepared by pooling acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid, and diluting the mixed standands with water to 0.1–500 μg $mL^{-1}$. The derivatization procedure was conducted as described previously [20].

GC–MS analysis was performed on an Agilent 7890B gas chromatography system coupled to an Agilent 5977C inert XL EI/CI mass spectrometric detector (MSD, Agilent Technologies, Santa Clara, CA) using an HP-5 ms capillary column (30 m × 250 μm i.d., 0.25 μm film thickness, Agilent J & W Scientific, Folsom, CA). The loading amount of derivative was 1 μL, the split ratio was 10:1, and the solvent delay time was 2.2 min. The initial oven temperature was maintained at 50 °C for 2 min, then gradually increased to 70 °C at the rate of 10 °C $min^{-1}$, to 85 °C at the rate of 3 °C $min^{-1}$, to 110 °C at the rate of 5 °C $min^{-1}$, to 290 °C at the rate of 30 °C $min^{-1}$, and finally maintained at 290 °C for 8 min. Helium was used as carrier gas and passed through the column at a constant flow rate of 1 mL $min^{-1}$. The temperature of inlet, transfer line, and electron impact (EI) ion source are set at 260 °C, 290 °C and 230 °C, respectively. The electron energy was −70 eV, and the mass spectrum data (M/Z 30-600) are collected in full scan mode.

GC–MS analysis software MSD Chemstation (version e.02.02.1431, Agilent Technologies, Inc.) was used to check the spectra, retention time, and characteristic mass nuclear ratio of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and internal standard (hexanoic acid-d3). MassHunter quantitative analysis software (version b.07.00) was used for quantitative analysis of single ion detection scanning data; the linear calibration curve was created by 6 standards and repeated injection for 3 times.

### 2.7. Detection of antibiotic resistance genes (ARGs)

GROOT was applied on the raw data to get the resistome profile for each sample [21], by mapping reads to specific antibiotic genes in the ARG-Annot database [22]. Thereafter, MetaCherchant was used to reconstruct the ARG-containing sequences for several major resistance genes [23]. Based on metagenomic data, MetaCherchant identified mobile elements-associated ARGs in bacterial genomes [23]. Finally, Kraken and its database was used to acquire the species with all the ARGs present in our sample [24].

### 2.8. Statistical analyses

Shannon index was computed to calculate alpha-diversity, which reflected the richness and evenness of each metagenomic sample, while Bray-Curtis dissimilarity distance was computed to estimate beta-diversity. Significant differences were evaluated by Wilcoxon tests between any two groups. Wilcoxon Signed-rank test was used to compare paired samples, and Wilcoxon Rank-sum test was used to compare samples of different groups. All P-values generated in our statistical analyses are listed in Tables S1. Differential abundant analysis of metagenomics data was performed using (linear discriminant analysis effect size) LEfSe [25], and *t* test with centred-log-ratio (CLR) transformation [26]. To avoid the zero-relative abundance in Equation, the zero abundance obtained by pseudo-counts of $1e^{-5}$ were replaced before relative abundance normalization and CLR transformation [27].

### 2.9. Data availability

Raw sequencing metagenome data have been made accessible in the NCBI Sequence Read Archive database under the Bioproject accession number PRJNA563204.

## 3. Results

### 3.1. The effect of antibiotic and probiotic on the growth of the rats

Changes in the rat body weight during the course of the experiment were monitored, and no significant difference was found between the experimental group (AB-Ctrl, AB-Prob, and AB + Prob) and the control group (Ctrl) during the entire intervention period (Fig S2), suggesting that the antibiotic or probiotic alone, as well as the combination of the two, did not significantly affect the growth of rats. Such results were consistent with that reported previously [28].

### 3.2. The effect of antibiotic and probiotic on the gut microbiota of rats

Samples from Ctrl at the four time points had similar alpha diversity while the other three treatment groups displayed dramatic decreases in alpha diversity on Day 7 (two-sided Wilcoxon rank-sum test, $p < 0.05$) when treated with cefdinir. However, AB + Prob group showed a significantly higher alpha-diversity than either the AB-Ctrl or AB-Prob groups (two-sided Wilcoxon rank-sum test, $p$ = 0.000010, 0.000043, respectively), suggesting that the probiotic treatment during/after antibiotic-based therapy was beneficial for partial recovery of intestinal microbiota (Fig. 1a). However, taking probiotic was not essential in full restoration of intestinal microbiota to the baseline level, as the alpha-diversity of AB-Ctrl group recovered without probiotic application after the course of antibiotic administration, suggesting that the microbiota community was able to recover naturally. Similar to the alpha-diversity, principal coordinate analysis (PCoA; Bray-Curtis dissimilarity distance) also showed that, at Day 7, the gut microbiota composition of rats received antibiotic treatment was significantly different from that of untreated samples (Day 0) in all three groups. The gut microbiota was gradually restored towards the baseline level at Day 21, and the trend of recovery of gut microbiota in AB-Prob and AB + Prob group was more obvious than that of AB-Ctrl group (Fig. 1b).

### 3.3. Distinct short-chain fatty acid (SCFA) profiles in probiotic and antibiotic treatment

The SCFA datasets were consistent with those of the metagenomics, where all the Day 7-antibiotic-treated samples had the same SCFAs composition. These results showed a drastic effect of antibiotic application on both the gut microbiota and SCFAs. Analytical SCFA quantification was performed at different time points for all sample groups to identify the key SCFAs significantly affected by the treatment groups.

A total of six SCFAs, namely acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid, were determined. Among them, four SCFAs were identified as being differentially abundant SCFAs by comparing data of AB-Ctrl group with those of AB-Prob group; and AB-Ctrl group with AB + Prob groups at Days 7, 14 and 21 (Fig. 2), trace or no isobutyric acid and isovaleric acid were detected among all samples (data not shown). The majority of the variance was driven by antibiotic treatment, which was consistent with the results of metagenomics analysis above. However, at Day 14, all samples tended to recover to baseline, and AB + Prob group showed better resilience. Especially, the level

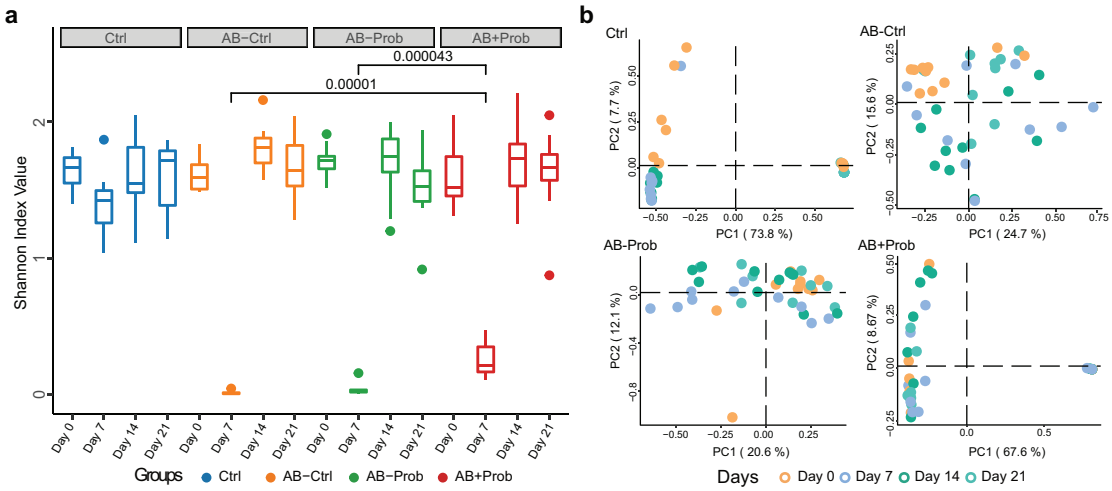


**Fig. 1.** Overview of alpha and beta diversity of metagenome datasets. (a) Alpha diversity of different groups: control received only saline (Ctrl), antibiotic (AB-Ctrl), antibiotic followed by probiotic treatment (AB-Prob), and antibiotic plus probiotic followed by probiotic (AB + Prob). The alpha-diversity (Shannon index) dropped dramatically in the AB-Ctrl, AB-Prob, and AB + Prob groups at Day 7; however, the Shannon index of AB + Prob group was significantly higher than the other two groups. (b) PCoA analysis showed the variation of gut microbiota composition and structure at different time points (Day 0, Day7, Day 14, and Day 21) in each group (Ctrl, AB-Ctrl, AB-Prob, and AB-Prob).

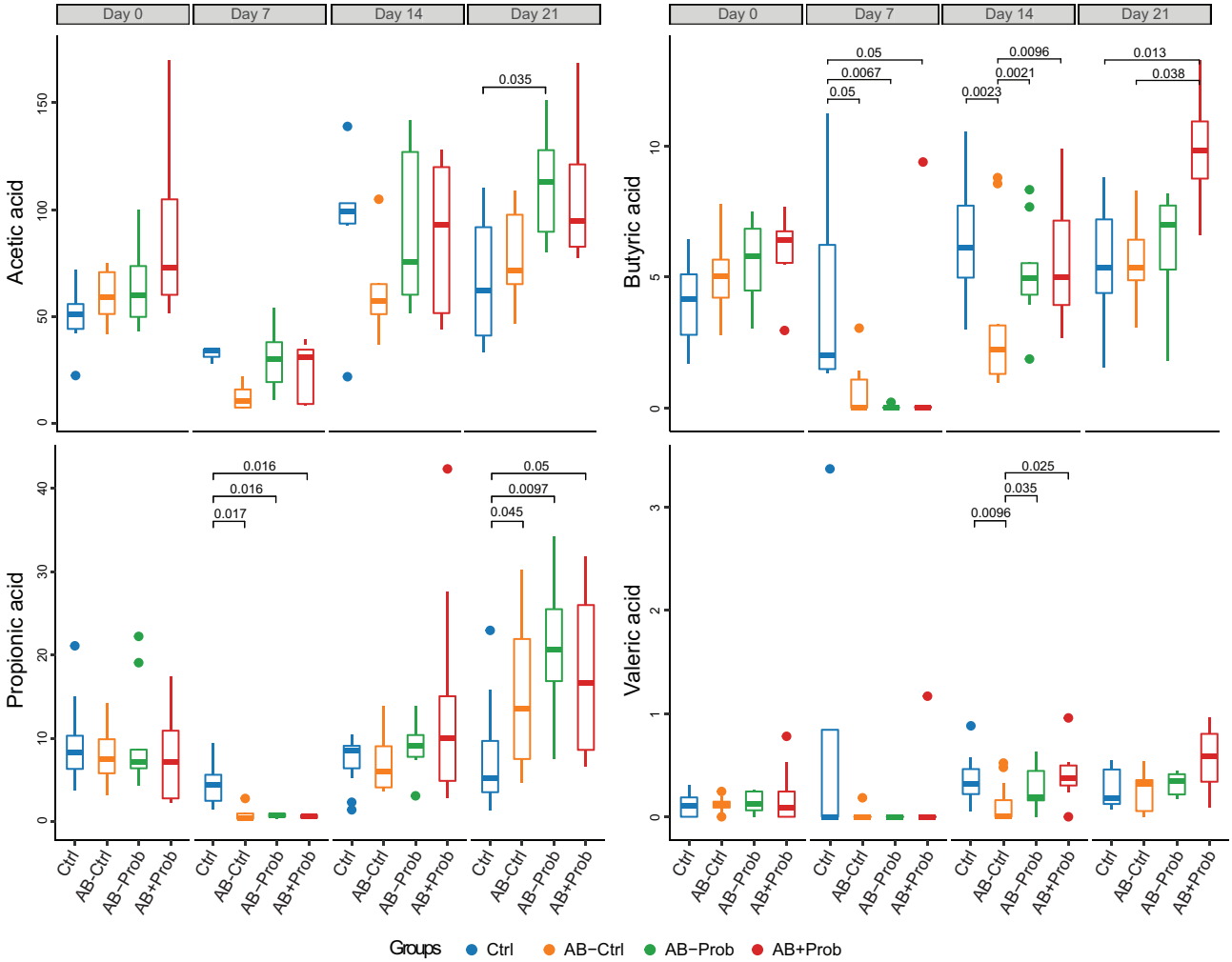


**Fig. 2.** Changes in short-chain fatty acids (SCFAs) in four groups during the course of experiment. The levels of SCFAs of different groups: control received only saline (Ctrl), antibiotic (AB-Ctrl), antibiotic followed by probiotic treatment (AB-Prob), and antibiotic plus probiotic followed by probiotic (AB + Prob). Only data of acetic acid, propionic acid, butyric acid, and valeric acid are presented, as other SCFAs (e.g., isobutyric acid and isovaleric acid) were present only in trace amount or undetected.

of butyric acid was resumed in an earlier time point for AB + Prob group compared with the other three groups (Fig. 2). On Day 21, the levels of propionic acid and butyric acid in AB + Prob group increased significantly compared with the Ctrl group, while the levels of acetic acid and propionic acid increased significantly in AB-Prob group compared with Ctrl group. In addition, at Day 14, the level of valeric acid in both AB + Prob and AB-Prob groups increased significantly compared with AB-Ctrl group. Altogether, the rapid resumption of SCFAs in the probiotic treated groups indicated that probiotic application could effectively facilitate the restoration of some key metabolic pathways of SCFAs.

### 3.4. Longitudinal analyses of the rat gut microbiota

Antibiotic-induced responses of the gut microbiota was analyzed by differential abundance analyses (LEfSe and T-test with CLR transformation), particularly by identifying species that disappeared soon after antibiotic treatment as well as those survived throughout. Analyses were performed by comparing the gut microbiota of AB-Ctrl, AB-Prob, and AB + Prob groups at different time points (Day 7 to Day 21). Drastic shift occurred in the gut microbiota composition during antibiotic treatment (first seven days), accompanied by an obvious increase in the relative abundance of the multidrug resistance species *Enterococcus faecium* [29] (Fig. 3b). In the end, 58 differentially abundant species were identified between AB-Ctrl and AB-Prob groups, and between the AB-Ctrl and AB+Prob group based on LEfSe analysis (Fig. S3–S8) and CLR transfer method. We also performed hierarchical clustering on species that had a relatively high prevalence in >20% of samples (Fig. S9). Our clustering results were consistent with the biological pathways and metabolic profile predicted using HUMAnN2 (Fig. S10) [30]. To further investigate the putative mechanism triggered by cefdinir and *L. casei* Zhang, we next determined the differentially abundant pathways between AB-Ctrl and AB-Prob groups as well as AB-Ctrl and AB + Prob groups at Day 7, Day 14, and Day 21. Family-level metagenomic changes were found to be significant after cefdinir treatment (at Day 7; Fig. 3a). Nevertheless, the microbiota composition returned to close to baseline by Day 14, which was consistent with our previous results (Fig. 1b). The major differentially abundant species in all probiotic-treated groups was *L. casei*. Except for the multi-drug resistant species, *Enterococcus faecium*, that fluctuated greatly with the use of antibiotics and probiotics, *P. goldsteinii* comprised the highest proportion among the differential abundant species. Moreover, based on the species-level microbiota composition (Fig. 3b), *P. goldsteinii* seemed to be an important species that contributed to the gut microbiota homeostasis in AB + Prob group. The species, *P. goldsteinii*, has been reported to reduce the levels of inflammation and insulin resistance, and several studies have reported that *P. goldsteinii* could be a potential next-generation probiotic [28,31,32]. In addition, *P. goldsteinii* was also significantly ($p$ = 0.034). Differential abundant in AB + Prob group versus AB-Ctrl group at Day 14 (Fig. 3c). Day 14 was the first time point after cefdinir intervention. Although the alpha diversity (Fig. 1a) and overall microbiota composition (Fig. 1b) seemed to have been recovered, the finer composition of at the species level, as well as changes in microbiome function, metabolite, and SCFAs, should be further investigated. Hence, the co-abundance between these two bacteria in the two probiotic-treated groups was analyzed, and strong correlations were found in both groups (AB-Prob group, R = 0.46, $p$ = 0.0031; AB + Prob group, R = 0.36, $p$ = 0.048). AB + Prob group received the probiotic continuously (with partial overlapping with antibiotic treatment), and since the antibiotic treatment would likely affect the bacterial composition, co-abundance analysis was not performed for data of AB + Prob group obtained at Day 7 (Fig. 3c).

### 3.5. Administering L. casei decreased ARG ratio

We attempted to quantify the proportions of ARG sequence by performing GROOT in fecal samples from all four groups. The ARG ratio increased significantly at Day 7 in AB-Ctrl, AB-Prob, and AB + Prob groups due to the cefdinir treatment (Fig. 4a), followed by a significant drop of the AGS ratio at Day 14, indicating the decrease in growth of AGS-possessing bacteria. AB + Prob group showed a decreasing trend until Day 21 (with marginal statistical significance, two-sided Wilcoxon signed-rank test, $p$ = 0.083). However, the ARG ratio of rats in all groups still remained at a high level at Day 21, with no significant difference found between treatment groups ($p$ > 0.05). The Ctrl group showed a significant difference in ARG ratio ($p$ = 0.0056) at Day 21 because of its intrinsically low level of ARG ratio. The comparatively higher levels of ARG ratio of the other three groups could have been a remaining effect of earlier cefdinir treatment. Next, we identified 19 ARGs using MetaCherchant, and the aminoglycosides resistance gene, AAC(6′)-APH(2″), seemed to be responsible for cefdinir resistance (Fig. 4b). AB + Prob group showed a larger decrease in AAC(6′)-APH(2″) gene. AAC(6′)-APH(2″) has previously only been detected in Gram-positive bacteria, including *Clostridioides* (*C.*) *difficile*, Enterococcus spp., *Burkholderiales* bacterium [33]. To further investigate the distribution of ARGs in the gut microbiota, we used Kraken to assign the remaining sequences taxonomically by mapping the raw reads to ARG-ANNOT, an ARG database. As expected, the mapped sequences were mostly assigned to the three bacterial taxa mentioned above. Significantly more *C. difficile* was detected in AB-Ctrl and AB-Prob groups than Ctrl group, while AB + Prob group had almost no *C. difficile* sequences at Day 14 (Fig. 4c). Cefdinir treatment led to the significant increase in the conditioned pathogen, *C. difficile*, in the rat gut microbiota. Although the rise in the gut opportunistic pathogens and ARGs was temporary and was eventually recovered in the rats naturally, the temporal increases in these potentially pathogenic elements in rat gut undoubtedly posed elevated risks of gastrointestinal diseases and spreading antibiotic resistance. Our data showed that probiotic treatment significantly lower such risks by maintaining the host's health and gut microbiota stability. Nevertheless, *Burkholderiales* bacterium also re-grew alone the recovery of gut microbiota, indicating that cefdinir could have long-term effects if not treated properly, while the relative abundance of *Burkholderiales* bacterium in AB + Prob group was closer to normal level compared to AB-Ctrl and AB-Prob group at Day 21 (Fig. 4d).

### 3.6. Possible associations between gut microbiota and SCFAs

We then identified differential microbial signatures and SCFAs that covaried across samples. Such relationships might give hints of the mechanism of how gut microbes modulated the host physiology via SCFAs production during cefdinir and *L. casei* Zhang treatments. For example, a positive SCFA-species association might suggest enhanced growth of a certain species by a SCFA or that the SCFA was indeed originated from this species. To identify such relationships, correlation analysis was done between previously identified differentially abundant SCFAs and microbial species. More importantly, we performed association discovery with Spearman's correlation analysis based on the abundance of SCFAs and species, which could exclude false positive represented merely mutually correlating pairs. Our results showed that the levels of SCFAs in AB + Prob and AB-Prob groups correlated negatively with species associated with AB + Prob and AB-Prob (Fig. 5a), such as multidrug resistance *Enterococcus faecium* and *Bifidobacterium longum*. Additionally, in all antibiotic-treated groups, the relative abundance of *P. goldsteinii* correlated strongly with propionic acid (Fig. 5b), butyric acid (Fig. 5c), and valeric acid (Fig. 5d). The species

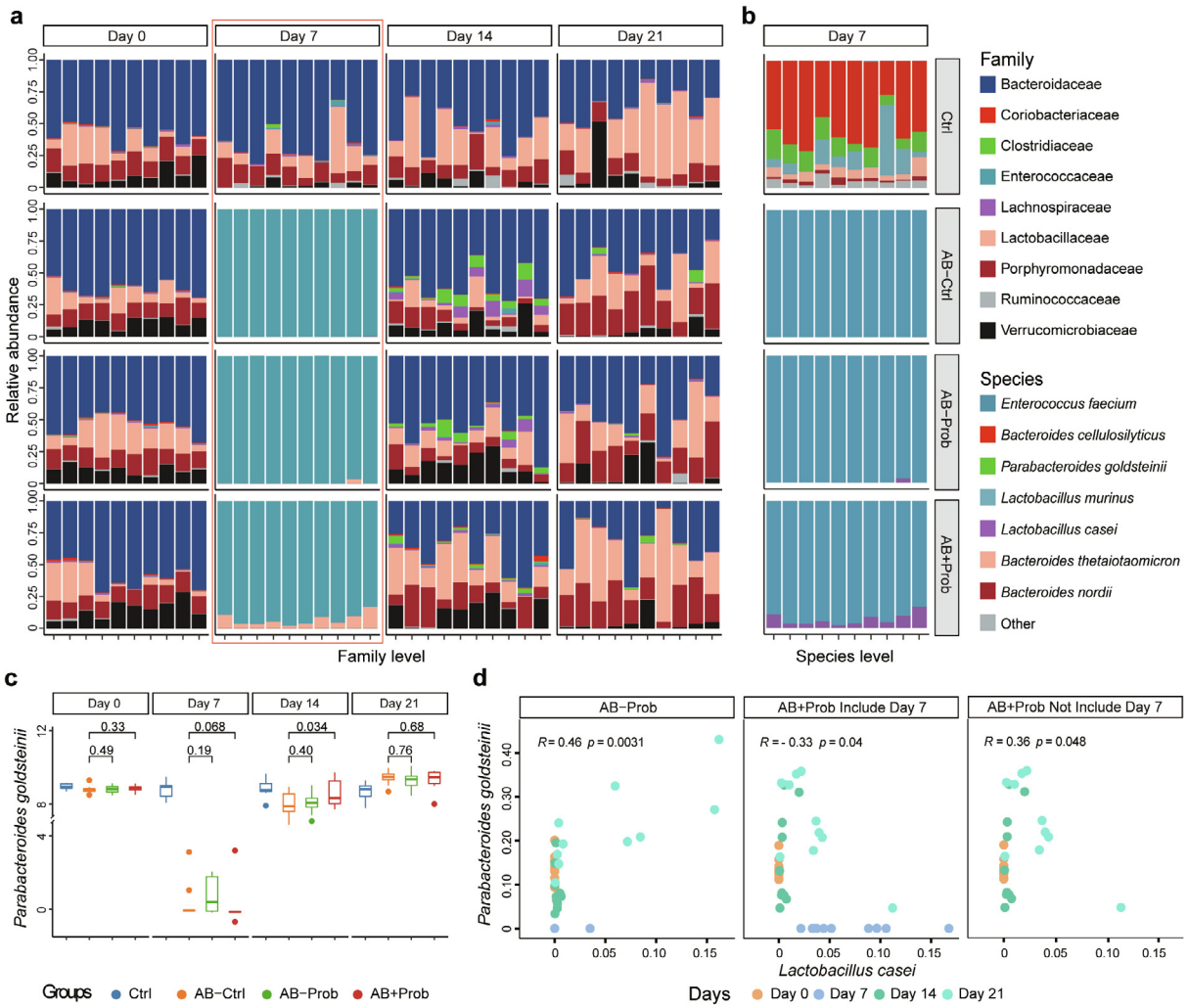


**Fig. 3.** Differentially abundant short-chain fatty acids (SCFAs) between groups were key factors that affected the chemical profile of each group. (a) Stacked bar plots of family-level phylogenetic composition of common bacterial taxa. The four experimental groups were control received only saline (Ctrl), antibiotic (AB-Ctrl), antibiotic followed by probiotic treatment (AB-Prob), and antibiotic plus probiotic followed by probiotic (AB + Prob). (b) Stacked bar plots of species-level phylogenetic composition of bacterial taxa at Day 7. (c) *Parabacteroides* (*P.*) *goldsteinii* was the significantly differential abundant species in AB + Prob group at Day 14. (d) *Lactobacillus casei* and *P. goldsteinii* correlated strongly in the metagenomic datasets of both AB-Prob and AB + Prob groups. The data of AB + Prob group at Day 7 are not shown due to possible interference of the antibiotic during the co-administration of antibiotic and probiotic.

has been reported to modulate the host circadian clock in the gastrointestinal tract [34], and these bacteria may regulate the host physiological responses via the three SCFAs.

### 3.7. Administering L. casei could mitigate gut dysbiosis and inflammation

The inflammatory effects caused by antibiotic treatment were then assessed by quantifying the abundance of cytokine markers in the rat serum samples. The cytokine expression was found to be associated with resident bacteria like *Clostridium*, *Clostridioides*, *Akkermansia* and *Lactobacillus* [35]. Therefore, the covariation between differentially abundant microbes and immune factors was also investigated. We focused on the IL-1 cytokine family because of its close link with the innate immunity (>95% of living organisms rely on innate immune mechanisms for survival and < 5% also employ T- and B-cell-based immunity) [36]. Hierarchical clustering with the correlation coefficients between immune factors and relative abundances of differential abundant bacteria across all groups at all four time points identified strong correlation between a gut microbial sub-population (mainly comprised members of *Clostridium* and *Clostridioides*, $0.15 < R < 0.3$, $p < 0.05$) and IL-1 family-related cytokines (Fig. 6a). Activated macrophages release IL-1α, triggering thymocyte proliferation via IL-2 secretion, B-cell maturation and proliferation, and activation of fibroblast growth factor [37]. Moreover, IL-1 proteins are endogenous inflammatory pyrogens that trigger prostaglandin and collagenase production from synovial cells. Thus, we evaluated IL-1α production in all four groups (Fig. 6b). The level of IL-1α in AB-Ctrl group increased monotonically from Day 7, suggesting that an inflammatory response was triggered by antibiotic intervention, but the probiotic treatment (both AB-Prob group and AB + Prob group) alleviated the inflammation. IL-1α correlated weakly and positively with the conditional pathogen *C. difficile* but negatively with *Bifidobacterium longum,* often found to be associated with a healthy state (Fig. 6c–e) [38].

## 4. Discussion

Using an integrated metagenomic, metabolomic, and immunological approach, we found that probiotic supplementation could alleviate cefdinir-induced gut dysbiosis. We sought to elucidate

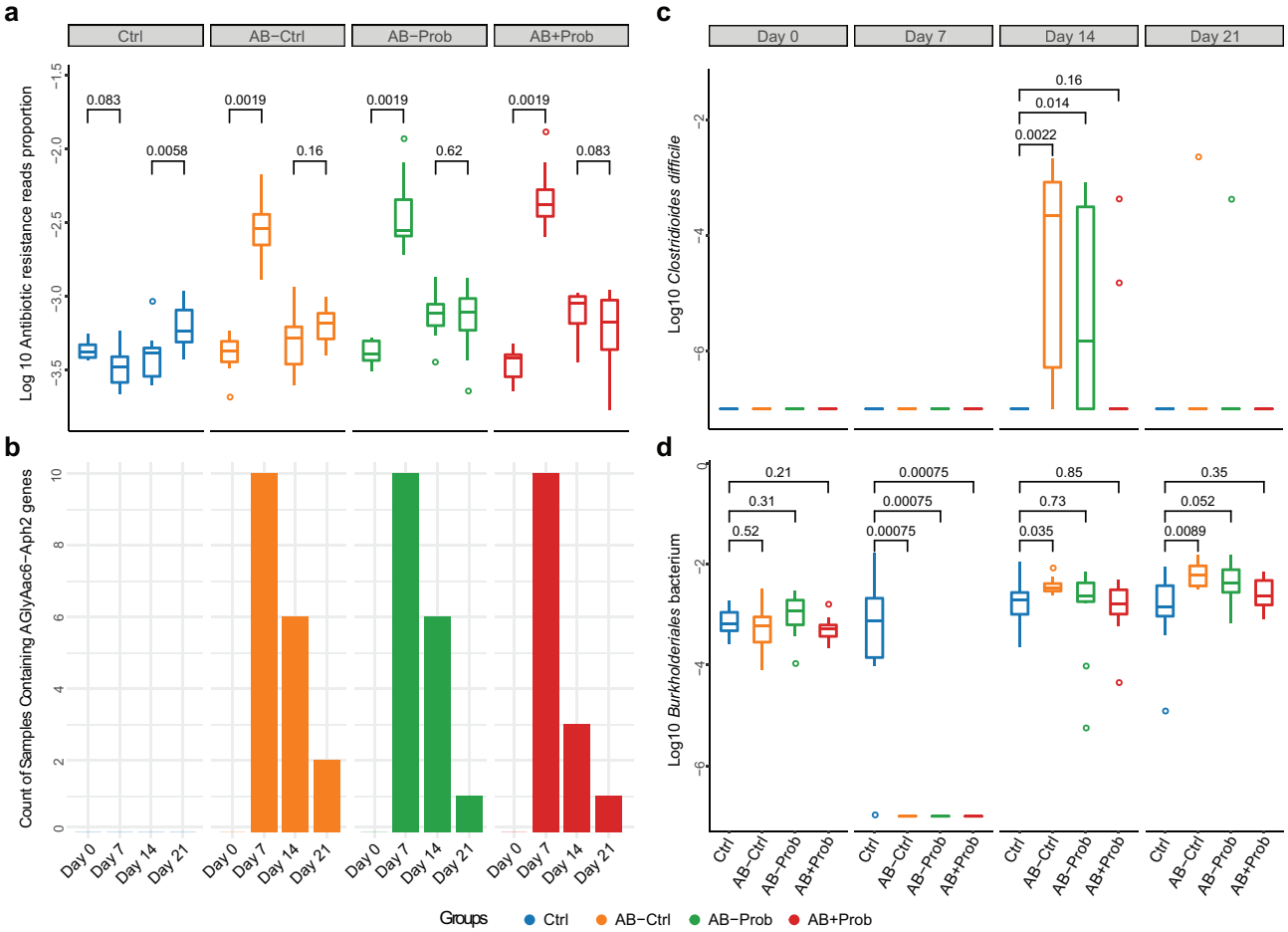


**Fig. 4.** Antibiotic resistance genes (ARGs) detected in all metagenomic datasets. The four experimental groups were control received only saline (Ctrl), antibiotic (AB-Ctrl), antibiotic followed by probiotic treatment (AB-Prob), and antibiotic plus probiotic followed by probiotic (AB + Prob). (a) The ratio of ARGs mapped reads was significantly higher in the AB-Ctrl, AB-Prob, and AB + Prob groups at Day 7, and the ratio of ARGs for the AB + Prob group showed a non-significant decreasing trend ($p = 0.083$). (b) AAC(6′)-APH(2″) genes detected in the four different groups at different time points. (c, d) Relative abundances of *Clostridioides difficile* and *Burkholderiales* bacterium.

the mechanism of such beneficial effects by evaluating the gut microbiota, SCFAs, and serum cytokine levels.

The most apparent effect of probiotic supplementation (both AB-Prob and AB + Prob) was the relative increase in *P. goldsteinii* and SCFAs, especially propionic acid and butyric acid. Moreover, the relative abundance of this potential probiotic species correlated strongly with the level of *L. casei*. The species, *P. goldsteinii*, has been reported to reduce expression of inflammatory cytokines, and several studies have reported that *P. goldsteinii* could be a potential next-generation probiotic [28,31,32]. Thus, it was interesting to see that the supplementation *L. casei* Zhang exerted anti-inflammatory effect to the rats, characterized by the reduction in inflammatory cytokines such as interleukin-1 [39]. However, the contribution of *P. goldsteinii* in the anti-inflammatory response remains to be further explored. The induction of inflammatory cytokines such as IL-1α was likely a harmful side effect in response to cefdinir intervention, and it continued until Day 21. Probiotic treatment (both AB-Prob group and AB + Prob group) obviously countered such harmful effect. Additionally, a weak positive correlation was observed between IL and 1α and the conditional pathogen *C. difficile*; while a weak but significant negative correlation was observed between IL and 1α and common beneficial bacteria *Bifidobacterium longum*, particularly in AB-Prob group. A potential reason could be that *L. casei* inhibited the growth of conditional pathogens via producing antimicrobial substances meanwhile enhancing the growth of beneficial bacteria. Although the mechanism of *L. casei* in inhibiting pathogens remains unclear, its effect on strengthening the host gut defense via competitive exclusion of bowel pathogens was previously reported [40]. Although no correlation was found between *C. difficile* and *Bifidobacterium longum* in AB + Prob group as in AB-Prob group, administering *L. casei* Zhang and cefdinir also significantly alleviated the antibiotic-induced inflammatory response. Yet, whether the anti-inflammatory effect of coadministration of antibiotic together with probiotic and supplementing probiotic only after antibiotic application was via identical mechanism also requires further investigation.

Acetic acid, propionic acid, butyric acid, and valeric acid are known to be the major components of SCFAs. Probably due to the widely distributed pathway of acetic acid synthesis, the amount of fecal or colonic acetic acid was found to be highest among SCFAs [41]. More than 90% of SCFAs are reabsorbed by the colon through protonation and anion exchange; they serve as energy sources or precursors for biosynthesis, and only 5–10% of SCFAs are excreted with feces [42]. After being transported to the liver, about 70% acetic acid is transformed into acetyl coenzyme A, which is the precursor of lipogenesis and gluconeogenesis. Most propionic acid reaches the liver through blood circulation to form propionate and then enters the glucose metabolism pathways. Butyric acid is commonly used by colonic epithelial cells to participate in colon mucosal hyperplasia and intestinal wall integrity maintenance, and only a small proportion is absorbed by intestinal

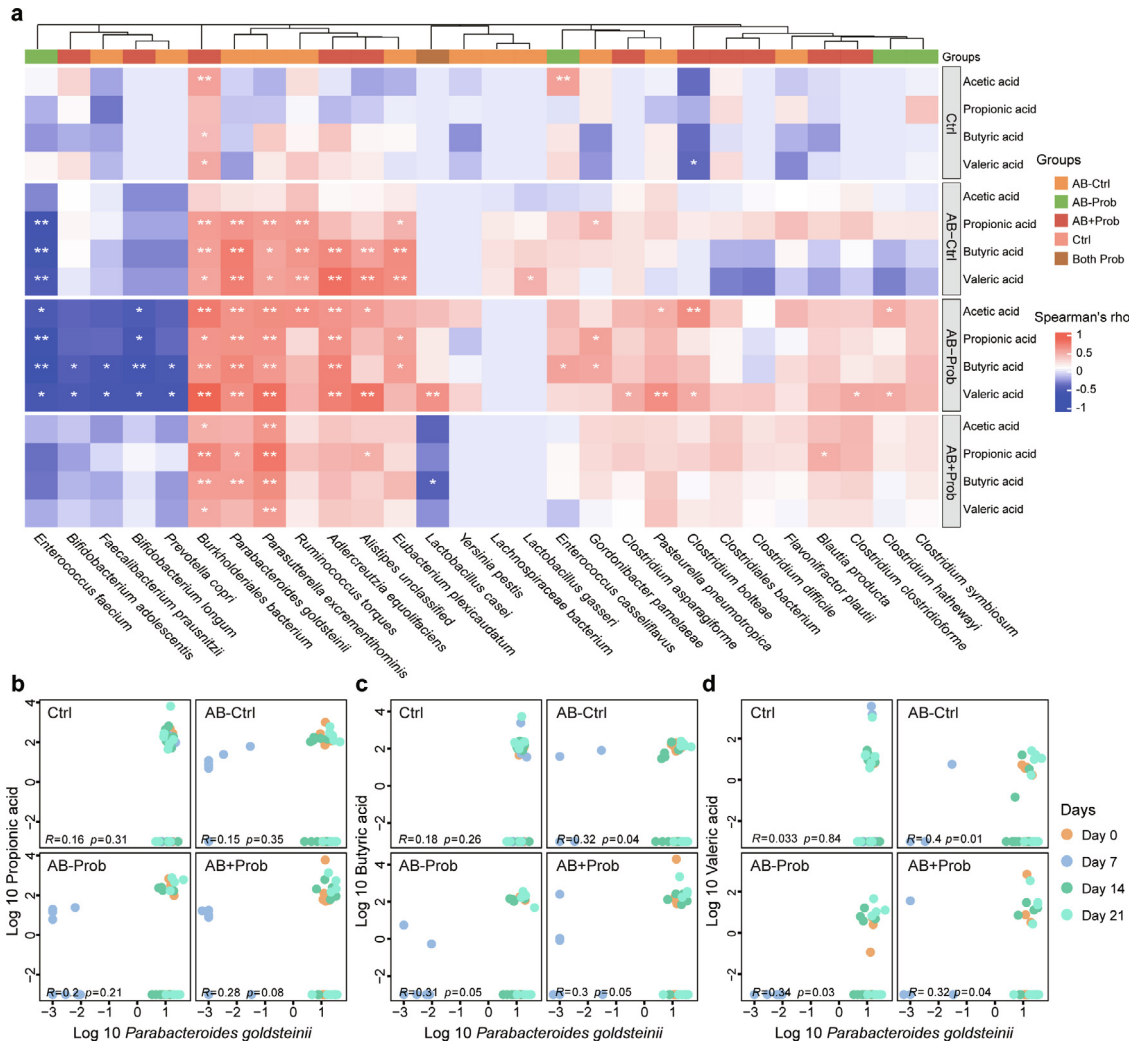


**Fig. 5.** Potential mechanistic associations between differential abundant gut microbes and short-chain fatty acids (SCFAs). The four experimental groups were control received only saline (Ctrl), antibiotic (AB-Ctrl), antibiotic followed by probiotic treatment (AB-Prob), and antibiotic plus probiotic followed by probiotic (AB + Prob). "Both Prob" represent data subsets of both probiotic treated groups. (a) Spearman's correlation between differential abundant gut microbes and SCFAs Significant differences are indicated by: * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$). (b, c, d) Correlation between three SCFAs and *Parabacteroides goldsteinii*.

epithelial cells into the circulatory system and transported to liver, heart, and lung [43]. Therefore, SCFAs have both systemic and local effects on the host to modulate host gastrointestinal health. It seems that ingesting probiotic after cefdinir intervention was more conducive to acetic acid production, which in turn effected on lipogenesis and carbohydrate synthesis, so as to stabilize the normal physiological functions of the rats. The co-feeding of cefdinir and probiotic was beneficial to butyrate synthesis, and the acid could promote colonic mucosal hyperplasia and enhance the integrity of the intestinal wall, meanwhile rebuilt the intestinal barrier. Regardless of when the probiotic was taken, propionate and valeric acid synthesis was promoted, and these acids improved the host's health. Remarkably, the levels of certain SCFAs in the two probiotic-receiving groups gradually recovered or even reached a higher magnitude after cefdinir treatment, and the restoration of SCFAs was positively correlated with *P. goldsteinii*. At the same time, *Parasutterella excrementihominis* may also played an important role in the probiotic effect of protecting rats from antibiotic-induced damage through SCFAs production. Probiotic-driven phase-dependent homeostasis of SCFAs has been reported to motivate intestinal immunomodulation [44]. Our results suggested that *L. casei* Zhang was beneficial for restoring host intestinal health in an antibiotic environment via reconstructing the SCFA-producing microbial subpopulation, and *P. goldsteinii* was likely an active contributor in such process.

We also investigated changes in ARGs during the course of intervention, and our results showed a significant decrease in the level of gut ARGs, and the decrease was observed sooner in the probiotic groups than the Ctrl group. It was likely that probiotic treatment reduced the microbiota subpopulation that contained the resistance genes, particularly the conditional pathogens, *C. difficile* and *Burkholderiales* bacterium. However, the species, *C. difficile*, was detected in the AB + Ctrl group only at Day 14, while the relative abundance of *Burkholderiales* bacterium decreased in AB + Prob group and returned to almost the baseline level. Pathogens and conditional pathogens are known to induce inflammation [45].

## 5. Conclusion

Antibiotic treatment not only led to host gut dysbiosis, but also induced both inflammatory responses and obvious increase in ARGs in the gut. Our results showed that daily intake of *L. casei* Zhang could be an effective way to facilitate the recovery of

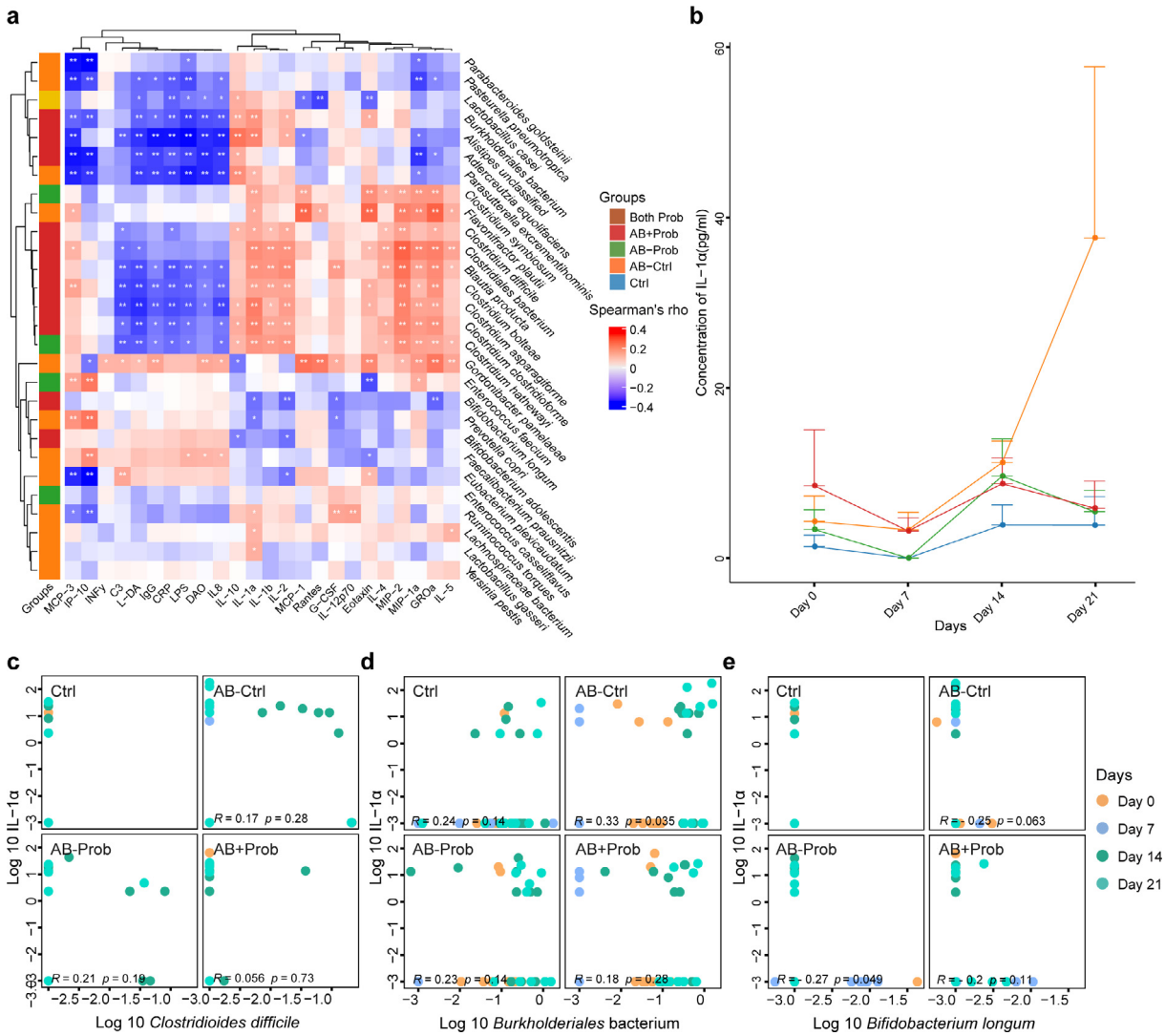


**Fig. 6.** Correlation between differential abundant gut microbes and immune factors. The four experimental groups were control received only saline (Ctrl), antibiotic (AB-Ctrl), antibiotic followed by probiotic treatment (AB-Prob), and antibiotic plus probiotic followed by probiotic (AB + Prob). (a) Spearman's correlation between differentially abundant microbiota and immune factors. Significant differences are shown: * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$). (b) Serum concentrations of interleukin (IL)-1α of all groups at all four time points. (c, d, e) Correlation between the serum interleukin (IL)-1α concentration and the relative abundance of *Clostridioides difficile*, *Burkholderiales* bacterium, and *Bifidobacterium longum*.

cefdinir-induced host gut dysbiosis in rats. As this strain is already on the market, its application could be easily adopted in clinical practice. The findings of this work provide important practical implications for understanding the role and mechanism of probiotics in protecting from gut dysbiosis caused by antibiotics. Moreover, the obtained data serve as evidence supporting the safe use of probiotics in clinical settings when antibiotics are concurrently applied.

## CRediT authorship contribution statement

**Guoqiang Yao:** Methodology. **Chenxia Cao:** Writing – original draft. **Meng Zhang:** Data curation, Visualization, Writing - review & editing. **Lai-Yu Kwok:** Writing – original draft, Writing - review & editing. **Heping Zhang:** Conceptualization, Supervision. **Wenyi Zhang:** Methodology, Supervision, Writing – original draft, Project administration. **:** .

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.10.026.

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