Characterising the Intestinal Bacterial and Fungal Microbiome Associated With Different Cytokine Profiles in Two *Bifidobacterium* strains Pre-Treated Rats With D-Galactosamine-Induced Liver Injury

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Multiple probiotics have protective effects against different types of liver injury. Different intestinal microbes could be beneficial to the protective effects of the probiotics on the treated cohorts in different aspects. The current study was designed to determine the intestinal bacterial and fungal microbiome associated with different cytokine profiles in the *Bifidobacterium pseudocatenulatum* LI09 and *Bifidobacterium catenulatum* LI10 pretreated rats with D-galactosamine-induced liver injury. In this study, partition around medoids clustering analysis determined two distinct cytokine profiles (i.e., CP1 and CP2) comprising the same 11 cytokines but with different levels among the LI09, LI10, positive control (PC), and negative control (NC) cohorts. All rats in PC and NC cohorts were determined with CP1 and CP2, respectively, while the rats with CP1 in LI09 and LI10 cohorts had more severe liver injury than those with CP2, suggesting that CP2 represented better immune status and was the “better cytokine profile” in this study.

PERMANOVA analyses showed that the compositions of both bacterial and fungal microbiome were different in the LI10 cohorts with different cytokine profiles, while the same compositions were similar between LI09 cohorts with different cytokine profiles. The phylotype abundances of both bacteria and fungi were different in the rats with different cytokine profiles in LI09 or LI10 cohorts according to similarity percentage (SIMPER) analyses results. At the composition level, multiple microbes were associated with different cytokine profiles in LI09 or LI10 cohorts, among which *Flavonifractor* and *Penicillium* were the bacterium and fungus most associated with LI09 cohort with CP2, while *Parabacteroides* and *Aspergillus* were the bacterium and fungus most associated with LI10 cohort with CP2. These microbes were determined to influence the cytokine profiles of the corresponding cohorts. At the structure level, *Corynebacterium* and *Cephalotrichiella* were determined as the two most powerful gatekeepers in the
microbiome networks of LI09 cohort CP2, while *Pseudoflavonitractor* was the most powerful gatekeeper in LI10 cohort with CP2. These identified intestinal microbes were likely to be beneficial to the effect of probiotic *Bifidobacterium* on the immunity improvement of the treated cohorts, and they could be potential microbial biomarkers assisting with the evaluation of immune status of probiotics-treated cohorts.

**Keywords:** intestinal microbiome, bacteria, fungi, probiotics, liver injury

## INTRODUCTION

Liver injury has caused great illness in human beings (1). It could be induced by multiple factors, e.g., drug, virus, alcohol, food additives, and dietary supplements (2–5). A variety of products and materials have been determined to have protective effects against different types of liver injury (6–8).

The protective effects of probiotics against liver injury have been widely reported. For example, *Lactobacillus plantarum* C88 was capable of preventing ethanol-induced mice liver injury (9). *Bacillus* spores could protect rats from acetaminophen-induced acute liver injury (10). *L. plantarum* C88 was found to protect mice from aflatoxin B1-induced liver injury (11).

Different intestinal microbes were likely to work in concert with probiotics to promote health. *Bifidobacterium pseudocatenulatum* LI09 and *Bifidobacterium catenulatum* LI10 were found to alleviate the severity of D-galactosamine (D-GalN)-induced rat liver injury (12). However, the intestinal microbes that can enhance the protective effects of the two *Bifidobacterium* on the improvement of cytokine profiles have not been determined.

The current study was designed to (1) characterize the intestinal bacterial and fungal microbiome associated with different cytokine profiles of LI09 and LI10 pretreated rats with D-GalN-induced liver injury and (2) investigate the microbes with the biomarker potentials to assist with the evaluation of better immune status in the probiotics-treated cohorts.

## MATERIALS AND METHODS

The animal experiments were performed as previously described (12), with a few modifications. Briefly, *B. pseudocatenulatum* LI09 and *B. catenulatum* LI10 were streaked on the trypticase–phytone–yeast agar and revived anaerobically at 37°C. The two bacterial strains were then prepared in physiological saline (PBS) at a final concentration of 3 × 10⁷ CFU/ml. Sprague–Dawley male pathogen-free rats weighting 250–350 g were fed with a standard laboratory rat chow and raised under the 12:12 light/dark cycle at 22°C for 7 days to adapt to the environment.

The 122 rats were randomly allocated into four cohorts, i.e., LI09 cohort (n = 40), LI10 cohort (n = 40), positive control (PC) cohort (n = 22), and negative control (NC) cohort (n = 20). The rats in LI09 and LI10 cohorts were orally administrated with a 1-ml aliquot of LI09 or LI10 (3 × 10⁷ CFU) for a week, while the rats in PC and NC cohorts were orally administrated with 1-ml aliquot of PBS for the same period. Afterwards, an intraperitoneal injection of D-GalN was given to each of the rats in LI09, LI10, and PC cohorts at a dose of 700 mg/kg body weight. Twenty-four hours after the induction of liver injury, all the living rats were anesthetized by an intraperitoneal injection of 10 mg/kg xylazine and 80 mg/kg ketamine, before being subjected to laparotomy for collection of the blood, liver, and cecal content. The study was approved by Animal Care and Use Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

### Measurement of Liver Function Variables

Serum was extracted from blood samples by centrifugation and stored at −80°C. Concentrations of liver function variables in serum, i.e., gamma glutamyl transferase (GGT), total bilirubin (TB), total bile acid (TBA), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), were measured by an automatic biochemical analyzer (Roche Diagnostics, Ottweiler, Germany) according to the manufacturer’s instructions.

### Measurement of Serum Cytokines

The concentrations of 23 cytokines in the serum samples were measured using a Bio-Plex Pro™ Rat Cytokine 23-Plex Assay kit (Bio-Rad Ltd., Hercules, CA, USA) as per the protocol of the manufacturer. These cytokines included macrophage inflammatory protein (MIP)-1α, MIP-3α, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, IL-18, monocyte chemotactant protein-1 (MCP-1), growth-related oncogene (GRO)/keratinocyte chemotactant (KC), regulated upon activation, normal T cell expressed, and secreted (RANTES), and vascular endothelial growth factor (VEGF).

### Evaluation of Liver Injury Severity

The liver tissue from the left liver lobe of each rat was dissected and fixed in 10% formalin solution, before being dehydrated and processed in paraffin using standard histological methods. The liver samples were stained and mounted on microscope slides. The liver injury severity was evaluated by a professional pathologist based on the Ishak scoring system (13).

### Molecular Experiments

DNA was extracted from the cecal samples by using a DNeasy PowerSoil kit (MoBio Laboratories Inc., Carlsbad, CA, USA). The extracted DNA was respectively amplified with bacterial
primers (i.e., 341F/785R) and fungal primers (i.e., ITS3F/ITS4R) (14, 15). The PCR products were purified by using a DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA, USA), and their concentrations were measured by using a Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The purified PCR products were submitted for sequencing on Illumina NovaSeq 6000 platform (Illumina Inc. USA).

**Processing of Sequencing Data**

The sequencing data were processed with standard bioinformatic procedures, e.g., merge, quality filter, singleton removal, and chimera check. The sequences were clustered into groups of amplicon sequence variants (ASVs). Bacterial phylotypes were classified using QIIME2 against the Silva 138 database, while fungal phylotypes were classified against the UNITE fungal database. One rat in the LI10 cohort was not recruited in the current study, as it did not provide enough sequencing reads. All the other rats were recruited for the subsequent analyses.

**Cytokine Profile Analyses**

One-way ANOVA tests and Mann–Whitney tests were used to determine the differences in 23 cytokines in LI09, LI10, PC, and NC cohorts. The cytokines with significant differences among the four cohorts were selected and transformed in log2(X+1) for the cytokine profile analyses.

Partition around medoids (PAM) clustering analysis was performed to cluster the cytokine profiles of all the four cohorts, after an average silhouette analysis being conducted to determine the optimal number of clusters. The four cohorts were determined with two distinct cytokine profiles, i.e., CP1 and CP2. The rats in LI09 and LI10 cohorts with two different cytokine profiles were defined as CP1_LI09, CP2_LI09, CP1_LI10, and CP2_LI10 cohorts.

**Comparisons of Liver Function Variables and Ishak Scores**

T-tests and Mann–Whitney tests were used to compare the liver function variables in CP1_LI09 and CP2_LI09 cohorts. The same tests were performed to compare the liver function variables in CP1_LI10 and CP2_LI10 cohorts.

Mann–Whitney tests were carried out to compare the Ishak scores of CP1_LI09 and CP2_LI09 and those of CP1_LI10 and CP2_LI10 cohorts.

**Bacterial and Fungal Microbiome Composition Analyses**

The alpha diversity indices (i.e., observed species and Shannon and Pielou indices) of the bacterial and fungal microbiome in the LI09 cohorts with different cytokine profiles, and their control cohorts (i.e., PC and NC cohorts), were all calculated. One-way ANOVA was used to compare the alpha diversity indices of the four cohorts, and t-tests were performed for the comparisons of LI09 and control cohorts. The same strategy was carried out for the same microbiome composition comparisons of LI10 cohorts with different cytokine profiles and their control cohorts.

Permutational analysis of variance (PERMANOVA) was conducted in R 4.1.0 to compare the CP1_LI09 and CP2_LI09 cohorts for their bacterial and fungal microbiome compositions and compare them with their control cohorts (i.e., PC and NC cohorts). The same strategy was performed for the same microbiome composition comparisons of CP1_LI10 and CP2_LI10 cohorts and their control cohorts.

Similarity percentage (SIMPER) analysis was performed to determine the bacterial and fungal microbiome similarities within the LI09 and LI10 cohorts with different cytokine profiles. The same analysis was used to determine the bacterial and fungal microbiome dissimilarities between CP1_LI09 and CP2_LI09 cohorts. The same strategy was used for the comparisons of the bacterial and fungal microbiome dissimilarities between CP1_LI10 and CP2_LI10 cohorts.

Linear discriminant analysis (LDA) effect size (LEfSe) was carried out to compare the bacterial and fungi microbiome of CP1_LI09 and CP2_LI09 cohorts, respectively, to determine the bacteria and fungi associated with each of the two cohorts. The same analysis was performed to determine the bacteria and fungi associated with CP1_LI10 and CP2_LI10 cohorts.

**Effects of Bacteria and Fungi on the Cytokine Profiles**

Spearman test was used to determine the individual correlations between the cytokines in the cytokine profiles and the microbes associated with CP1_LI09, CP2_LI09, CP1_LI10, and CP2_LI10 cohorts.

Distance-based redundancy analysis (db-RDA) was performed to determine the effect of bacteria and fungi associated with CP1_LI09, CP2_LI09, CP1_LI10, and CP2_LI10 cohorts on the corresponding cytokine profiles.

**Correlations Between Bacteria and Fungi**

Spearman test was carried out to investigate the correlations between the bacteria and fungi associated with CP1_LI09 cohort. The same strategy was performed for the bacteria and fungi correlations in CP2_LI09, CP1_LI10, and CP2_LI10 cohorts.

**Microbiological Network Analyses**

The correlations between the bacteria and fungi in the intestinal microbiome networks of LI09 and LI10 cohorts with different cytokine profiles were determined by co-occurrence network inference (CoNet) analysis. Five metrics, i.e., Bray–Curtis, Spearman, Pearson, mutual information, and Kullback–Leibler dissimilarities, were used to calculate the ensemble inference in CP1_LI09, CP2_LI09, CP1_LI10, and CP2_LI10 cohorts. The top 10 microbes (i.e., bacteria and fungi) with the largest numbers of correlations in the microbiome networks of the four cohorts were determined.

Network fragmentation calculations and generation of a null distribution were carried out in R as previously described (16) to explore the gatekeeper(s) in the microbiome networks of LI09 and LI10 cohorts with different cytokine profiles. Statistical significance was defined as the number of times a fragmentation score over that resulting from the removal of the bacteria or fungi observed within the null distribution.
RESULTS

Cytokine Profile Analysis

Eleven serum cytokines were determined with significant differences among the LI09, LI10, PC, and NC cohorts, i.e., IL-1α, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-17A, M-CSF, MCP-1, MIP-3α, and RANTES (all p < 0.03), and they were used for the subsequent clustering analysis of cytokine profiles. Higher levels of IL-1α and M-CSF were determined in both LI09 and LI10 cohorts than in PC cohort, and IL-2 and IL-17A were greater in the LI10 cohort than in the PC cohort (Supplementary Figure S1), suggesting IL-1α, M-CSF, IL-2, and IL-17A were enhanced by LI09 and/or LI10. In contrast, MCP-1, IL-5, and MIP-3α were determined with lower levels in both LI09 and LI10 cohorts than in the PC cohort (Supplementary Figure S1), suggesting that they were suppressed by LI09 and LI10.

Two was determined as the most optimal number for clustering the cytokine profiles (Figure 1A), and two distinct cytokine profiles (i.e., CP1 and CP2) were determined in the LI09, LI10, PC, and NC cohorts (Figure 1B). All rats in the PC cohort were determined with CP1, and all rats in the NC cohort had CP2, suggesting that CP2 represented better immune status and was the “better cytokine profile.” Twenty-one rats in the LI09 cohort and 18 rats in the LI10 cohort were determined with CP1, while 19 rats in the LI09 cohort and 21 rats in the LI10 cohort had CP2 (Figure 1B).

Liver Function and Liver Injury Severity Analyses

Six out of the seven measured liver function variables, i.e., ALT, AST, ALP, TBA, TB, and GGT, were greater in the CP1_LI09 cohort than in the CP2_LI09 cohort (Figure 2). By contrast, ALB was at similar level between the two cohorts.

Similarly, ALT, AST, ALP, TBA, TB, and GGT were determined at higher levels in the CP1_LI10 cohort than in the CP2_LI10 cohort (Figure 3), while ALB was lower in the CP1_LI10 cohort than in the CP2_LI10 cohort (Figure 3).

Ishak scoring system was used to help evaluate the liver histopathology, and in this study, a higher Ishak score represented greater liver injury severity. The Ishak score was greater in the CP1_LI09 cohort than in the CP2_LI09 cohort (Figure 4A), and the same score was greater in the CP1_LI10 cohort than in the CP2_LI10 cohort (Figure 4B). The rats with CP1 in LI09 and LI10 cohorts had more severe liver injury than those with CP2, further suggesting that CP2 was the “better cytokine profile” compared with CP1.

Bacterial Microbiome Composition Analyses

Firmicutes, Bacteroidota, and Verrucomicrobiota were determined as the three most abundant bacterial phyla in the LI09 and LI10 cohorts with different cytokine profiles.

At the family level, Lachnospiraceae and Bacteroidaceae were determined with the largest abundances in the bacterial microbiome of all the LI09 and LI10 cohorts with different cytokine profiles (Figure 5). Akkermansiacae was the third most abundant bacterial family in CP1_LI09, CP1_LI10, and CP2_LI09 cohorts, while Tannerellaceae was determined with the third most abundance in CP2_LI10 cohort (Figure 5).

Shannon and Pielou indices were both similar in bacterial microbiome of the CP1_LI09, CP2_LI09, PC, and NC cohorts (both p > 0.09), while a significant difference was determined in observed species of the four cohorts (p < 0.001). CP1_LI09 and CP2_LI09 cohorts were found with similar observed species, while they were both greater than in the NC cohort (Supplementary Figure S2A). Likewise, Shannon and Pielou indices were both similar between CP1_LI10, CP2_LI10, PC, and NC cohorts (both p > 0.05), while observed species was different between the four cohorts (p < 0.001). No difference was found in the observed species of CP1_LI10 and CP2_LI10 cohorts, but they were both greater than in the PC and NC cohorts (Supplementary Figure S2B).

PERMANOVA analysis suggested that the bacterial composition was similar between CP1_LI09 and CP2_LI09 cohorts (R² = 0.028, p > 0.26), and they were both different from the PC and NC cohorts (p < 0.002) The same analysis determined a significant difference in the bacterial composition between CP1_LI10 and CP2_LI10 (R² = 0.063, p < 0.001), and they were both different from the PC and NC cohorts (p < 0.001). SIMPER analysis determined that the similarity of bacterial phylotype abundances within CP1_LI09 was higher than that of CP2_LI09, i.e., 51% versus 46%. The same analysis determined a dissimilarity of 52% between CP1_LI09 and CP2_LI09 cohorts. Likewise, SIMPER analysis determined that the similarity of bacterial phylotype abundances was greater within CP1_LI10 (i.e., 51%) than within CP2_LI10 (i.e., 44%). The same analysis determined a dissimilarity of 55% between CP1_LI10 and CP2_LI09 cohorts.

LEfSe analysis determined five bacteria associated with CP1_LI09 cohort and one bacterium (i.e., Flavonifractor) associated with CP2_LI09 cohort (Figure 6A), among which Lachnospiraceae_UCG_006 was most associated with CP1_LI09 cohort. The same analysis revealed that 30 bacteria were associated with CP1_LI09 and CP2_LI10 cohorts (Figure 6B), among which Lachnospiraceae_NK4A136_group and Parabacteroides were most associated with CP1_LI10 and CP2_LI10 cohorts, respectively.

Fungal Microbiome Composition Analyses

Ascomycota and Basidiomycota were determined as the two most abundant fungal phyla in all the LI09 and LI10 cohorts with different cytokine profiles.

At the family level, Aspergillaceae and Trichocomaceae were determined with most abundances in the LI09 and LI10 cohorts with different cytokine profiles (Figure 7). Debaryomycetaceae, Hypocreales_Incertae_sedis, Trichosphaeriaceae, and Mrajaceae were determined as the third largest abundances in the mycobiome of CP1_LI09, CP1_LI10 and CP2_LI09 and CP2_LI10 cohorts, respectively (Figure 7).

Shannon and Pielou indices were both similar in the mycobiome between the CP1_LI09, CP2_LI09, PC, and NC cohorts (both p > 0.68), while observed fungal species was different in the four cohorts (p < 0.009). No difference was
determined in the fungal observed species of CP1_LI09 and CP2_LI09, but they were both less than that in the NC cohort (Supplementary Figure S3). Likewise, the three alpha diversity indices were all similar in the mycobiome of CP1_LI10, CP2_LI10, PC, and NC cohorts (all $p > 0.53$).

PERMANOVA analysis revealed that the mycobiome composition was similar between CP1_LI09 and CP2_LI09 cohorts ($R^2 = 0.03, p > 0.22$), but they were both different from PC and NC cohorts ($p < 0.004$). The same analysis determined a significant difference in the mycobiome composition between CP1_LI10 and CP2_LI10 cohorts ($R^2 = 0.049, p < 0.007$), and they were both different from PC and NC cohorts ($p < 0.025$).

SIMPER analysis determined that the similarity of fungal phylotype abundances within the CP1_LI09 cohort (i.e., 29%) was lower than that within the CP2_LI09 cohort (i.e., 34%). The same analysis determined a dissimilarity of 69% between CP1_LI09 and CP2_LI09 cohorts.

The same analysis determined that the similarity of fungal phylotype abundances within the CP1_LI10 cohort (i.e., 34%) was lower than that within the CP2_LI10 cohort (i.e., 40%). The same analysis determined a dissimilarity of 65% between CP1_LI09 and CP2_LI09 cohorts.

LEfSe analysis determined seven fungi associated with CP1_LI09 and CP2_LI09 cohorts (Figure 8A), among which Meyerozyma and Penicillium were most associated with CP1_LI09 and CP2_LI09 cohorts, respectively. The same analysis determined that 12 fungi were associated with CP1_LI10 and CP2_LI10 cohorts (Figure 8B), among which Talaromyces and Aspergillus were associated with CP1_LI10 and CP2_LI10 cohorts, respectively.

**Effects of Bacteria and Fungi on the Cytokine Profiles**

Multiple correlations were determined between the cytokines in cytokine profile and the microbes associated with each of the CP1_LI09, CP1_LI10, and CP2_LI10 cohorts, except the CP2_LI09 cohort (Figure 9). ASF356 and Meyerozyma were determined with more correlations in the CP1_LI09 cohort (Figure 9A). Lachnospiraceae_NK4A136_group, Meyerozyma, Stemphylium, and Talaromyces were determined with more
correlations with the cytokines in the CP1_LI10 cohort, and Talaromyces seemed to have an opposite effect on IL-α and M-CSF when comparing with Meyerozyma and Stemphylium (Figure 9C). GCA_900066575 was negatively correlated with most cytokines in the cytokine profile of CP2_LI10 cohort (Figure 9D).

Some of the bacteria and fungi closely associated with CP1_LI09, CP2_LI09, CP1_LI10, and CP2_LI10 cohorts were determined to influence the cytokine profiles in the corresponding cohorts (Figure 10).

Correlations Between Bacteria and Fungi

Different correlations were determined between the bacteria and fungi associated with CP1_LI09, CP2_LI09, CP1_LI10, or CP2_LI10 cohorts. Three correlations were determined in the bacterial and fungi associated with the CP1_LI09 cohort, i.e., a positive correlation between Prevotellaceae_UCG_001 and Meyerozyma, negative correlations between Lachnospiraceae_UCG_006 and Alternaria and between ASF356 and Issatchenka. By contrast, no correlation was determined between the bacteria and fungi associated with the CP2_LI09 cohort.

All the nine correlations were determined to be positive between six bacterial and six fungi associated with the CP1_LI10 cohort (Figure 11). Defluvitaleaceae_UCG_011, Candidatus_Christensenellaceae, and Pygmaiobacter were positively correlated with Oidiodendron in the CP2_LI10 cohort.

Microbiological Network Analyses

CoNet results revealed 10 microbes with most correlations in the intestinal microbiome networks of LI09 and LI10 cohorts with different cytokine profiles (Tables 1, 2). Seven out of the top 10 microbes with most correlations in the CP2_LI09 cohort were not determined in the top 10 microbes in the CP1_LI09 cohort (Table 1). Likewise, the top eight microbes with most correlations in the CP1_LI10 cohort were all not determined in
the top 10 microbes in the CP2_LI10 cohort (Table 2). *Eubacterium* and *Clostridium* were both determined with many correlations in the microbiome networks of both CP2_LI09 and CP2_LI10 cohorts but not in the probiotics-treated cohorts with CP1.

Among these bacteria and fungi, *Hungatella*, *Papillibacter*, and *Myceliophthora* were determined as gatekeepers in the network of the CP1_LI09 cohort (fragmentation analyses, all \( p < 0.05 \)). In the CP2_LI09 cohort, *Corynebacterium*, *Eubacterium*, *Papillibacter*, and *Cephalotrichiella* were identified as the microbiome network gatekeepers (fragmentation analyses, all \( p < 0.05 \)). By contrast, *Udeniomyces* and *Pseudo flavonifractor* were determined as the only gatekeepers in the microbiome networks of CP1_LI10 and CP2_LI10 cohorts, respectively (fragmentation analyses, both \( p < 0.05 \)).

Fragmentation analysis showed that the fragmentation level of the microbiome network of CP1_LI09 cohort was lower than that of the CP2_LI09 cohort, i.e., 0.497 versus 0.575. Similarly, the fragmentation level was lower in the CP1_LI10 cohort (i.e., 0.416) than that in the CP2_LI10 cohort (i.e., 0.527).

**DISCUSSION**

Multiple probiotics have been used to alleviate different types of liver injury (17–19). Liver injury severity, gut microbiome alterations, changes in liver function, and cytokine variables have been used to evaluate the effects of probiotics on the treated cohort (20, 21). Different intestinal microbes could have different abilities of improving these different aspects of the probiotics-treated cohorts. The present study demonstrated the intestinal microbiome associated with distinct cytokine profiles of the LI09 and LI10 pre-treated rats with liver injury and explored the microbes with the biomarker potentials to assist with the evaluation of better immune status in the two probiotics-treated cohorts.
Cytokine profiles have been investigated in the cohorts treated with probiotics (22, 23). In this study, the two cytokine profiles (i.e., CP1 and CP2) were determined by PAM clustering analysis based on the overall pattern of the same 11 cytokines, which were determined with significant different levels among LI09, LI10, PC, and NC cohorts. All rats in PC and NC cohorts were determined with CP1 and CP2, respectively, while the rats with CP1 in LI09 and LI10 cohorts were determined with more severe liver injury than those with CP2, suggesting that CP2 represented better immune status and was the “better immune profile” compared with CP1. PAM analysis has been widely used in the microbiome studies to cluster the microbiome in the same or different cohorts (24, 25) but was seldom used to cluster the immune variables.

The cytokines in the cytokine profile in this study, i.e., IL-1α, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-17A, M-CSF, MCP-1, MIP-3α, and RANTES, were all important to the immune system of mammals (26, 27). Their alterations were associated with the cohorts with different types of liver injury. T-cell activation could be promoted by IL-1α in mice with carbon tetrachloride-induced liver injury (28). IL-2 and IL-17A were independent risk factors that could lead to liver injury in coronavirus disease 2019 (COVID-19) patients at admission (29). Elevated level of plasma IL-4 was determined in the mice with dicloxacillin-induced liver injury (30). Serum IL-5 was increased in the mice with Schistosoma mansoni-induced granulomatous liver injury (31). The increased level of serum IL-6 was found in the rats with thioacetamide-induced liver injury (32). DEREG mice with liver fibrosis were found with higher level of IL-12p70, MCP-1, and RANTES (33). M-CSF and MIP-3α were increased in the rats with D-GalN-induced acute liver injury (12).

The majority of the measured liver function variables were determined at higher levels in the LI09 and LI10 cohorts with CP1 (i.e., CP1_LI09 and CP1_LI10 cohorts) than the corresponding cohorts with CP2 (i.e., CP2_LI09 and CP2_LI10 cohorts). As previous study has determined that the majority of
liver function variables were lower in the NC cohort than in the PC cohort (12), it suggested that the cohorts with CP2 had better liver function compared with those with CP1. Similarly, Ishak score was greater in CP1_LI09 and CP1_LI10 cohorts than the corresponding cohorts with CP2. As lower Ishak score represented lower liver injury severity (34, 35), it indicated that the cohorts with CP2 profile were with lower liver injury severity.

Bacterial microbiome alterations in probiotics-treated cohorts have been well studied (36, 37). In the current study, PERMANOVA results showed that the compositions of both bacterial and fungal mycobiome were similar between the CP1_LI09 and CP2_LI09 cohorts, while the same microbial compositions were different between the CP1_LI10 and CP2_LI10 cohorts. These suggest that the better cytokine profile in the LI10 cohort were associated with the altered bacterial and fungal microbiome compositions. SIMPER analysis has been widely used in the microbiome studies for different objectives (38, 39). SIMPER analyses revealed that the phylotype abundances of bacterial and fungal microbiome were both different between CP1_LI09 and CP2_LI09 cohorts, and between CP1_LI10 and CP2_LI10 cohorts, suggesting that the different cytokine profiles in LI09 and LI10 cohorts were associated with the altered phylotype abundances of the intestinal microbiome.

LEfSe has been carried out in multiple microbiome studies to determine the phylotypes associated with the different cohorts (40, 41). LEfSe analysis determined that multiple bacteria were associated with LI09 cohorts with different cytokine profiles, among which Lachnospiraceae_UCG_006 and Flavonifractor were most associated with CP1_LI09 and CP2_LI09 cohorts, respectively. The enriched Lachnospiraceae_UCG_006 was associated with the intervention of Nostoc commune Vaucher by polysaccharides (42). Flavonifractor plautilii was capable of attenuating inflammatory responses in the obese adipose tissue (43). As for fungi, Meyerozyma and Penicillium were most associated with CP1_LI09 and CP2_LI09 cohorts, respectively. Meyerozyma has been found in the patients with vulvovaginal candidiasis infection, while some Penicillium species have been used for the beneficial products and cheese-making (44, 45). Some alternative fungi were also closely associated with CP2_LI09 cohort, e.g., Sporobolomyces and Fusicolla. Sporobolomyces could accumulate beneficial metabolites (46), while Fusicolla was found as a fungus of soil origin (47).

Likewise, multiple bacteria and fungi were associated with the LI10 cohorts with different cytokine profiles. Lachnospiraceae_NK4A136_group and Talaromyces were the bacterium and fungus most associated with the CP1_LI10 cohort, while Parabacteroides and Aspergillus were the bacterium and fungus most associated with the CP2_LI10 cohort. Lachnospiraceae_NK4A136_group was determined with low abundance in the obese mice (48), while Talaromyces has been found as pathogenic fungus in human beings (49). Parabacteroides was a commensal gut bacterium and has been used to alleviate 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice (50). Aspergillus has been found to produce beneficial protease to improve the colonic luminal environment in rats with high-fat diet (51). Some alternative bacteria and fungi were also closely associated with CP2_LI10

![Figure 5](https://example.com/figure5.png)
cohort, e.g., Pygmaibacter, GCA_900066575, and Oidiodendron. More abundant Pygmaibacter was found in the intestines of type 2 diabetes mice treated with debranched corn starch (52). Increased gut GCA_900066575 has been determined in the high-fat diet mice (53), while Oidiodendron could improve the root biomass of Vaccinium corymbosum (54).

The effect of clinical variables or environmental factors on the microbiome has been well reported (24, 55). However, the potential effect of microbe on the immune profile was seldom studied. In the current study, although the bacteria and fungi associated with each of the four cohort (i.e., CP1_LI09, CP2_LI09, CP1_LI10, and CP2_LI10) were determined to have varied correlations with the individual cytokines, there was no obvious difference in the correlation patterns between the CP1 and CP2 in LI09 and LI10 cohorts. However, the db-RDA results revealed that the microbes associated with each of the four...
FIGURE 7 | Fungal family compositions in the LI09 and LI10 cohorts with different cytokine profiles, i.e., (A) CP1_LI09, (B) CP2_LI09, (C) CP1_LI10 and (D) CP2_LI10 cohorts.

FIGURE 8 | LEfSe analysis determined the fungi associated with (A) LI09 cohorts with different cytokine profiles (i.e., CP1_LI09 and CP2_LI09 cohorts) and (B) LI10 cohorts with different cytokine profiles (i.e., CP1_LI10 and CP2_LI10 cohorts).
Distance-based redundancy analyses revealed the impact of bacteria associated with (A) CP1_LI09 and CP2_LI09 and (B) CP1_LI10 and CP2_LI10 cohorts on the cytokine profiles of the corresponding cohorts, and the impact of fungi associated with (C) CP1_LI09 and CP2_LI09, and (D) CP1_LI10 and CP2_LI10 cohorts on the cytokine profiles of the corresponding cohorts. Up to five bacteria or fungi most associated with each cohort were selected for this analysis.

Correlations between cytokines in the cytokine profile and the microbes associated with each of (A) CP1_LI09, (B) CP2_LI09, (C) CP1_LI10, and (D) CP2_LI10 cohorts. “B” and “F” represent bacteria and fungi, respectively. Cross represents no positive or negative correlation.
cohort seemed to influence the corresponding cytokine profiles and were likely to be associated with the formation of distinct cytokine profiles. We acknowledge that the detailed mechanisms of the effects of microbes on the cytokine profiles need further investigation.

The correlations between bacteria and fungi were determined in multiple studies for different objectives (56, 57). In this study, different correlations were determined in the LI09 and LI10 cohorts with different cytokine profiles, but it seemed that no obvious difference was found in the correlation patterns or types (i.e., positive and negative) between the different cytokine profiles in the same cohorts.

CoNet and fragmentation analyses have been used to investigate the microbiome networks in multiple studies (16). Corynebacterium, Eubacterium, Papillibacter, and Cephalotrichiella were identified as the microbiome network gatekeepers in the CP2_LI09 cohort, while Pseudoflavonifractor was the only gatekeeper in the CP2_LI10 cohort. Some Corynebacterium and Eubacterium species have been determined to have beneficial potentials (58, 59). Papillibacter was determined to have the potential to assist Enterococcus faecium in enhancing the absorption and utilization of phosphorus (60). Pseudoflavonifractor was associated with the regulation of inflammation response in the aged mice with Listeria monocytogenes infection (61).

Lower fragmentation levels in the microbiome indicate greater co-occurrence patterns and more biotic interactions (16). The network fragmentation levels of LI09 and LI10 cohorts with CP1 were lower than the corresponding cohorts with CP2, suggesting that more biotic interactions were in the LI09 and LI10 cohorts with CP1 than those with CP2. This could be partly supported by the finding that there are more correlations between bacteria and fungi in the LI09 and LI10 cohorts with CP1 than in the corresponding cohorts with CP2.

In conclusion, the intestinal microbiome associated with distinct cytokine profiles in LI09 and LI10 pre-treated rats with liver injury was characterized. Multiple bacteria and fungi were associated with the better cytokine profiles in LI09 and LI10

### TABLE 1

The top 10 microbes with most correlations in the intestinal microbiome networks of the LI09 cohorts with different cytokine profiles, i.e., CP1_LI09 and CP2_LI09 cohorts.

| Rank | CP1_LI09 cohort | CP2_LI09 cohort |
|------|----------------|----------------|
| 1    | B_Papillibacter | B_Intestinalmonas |
| 2    | B_Corynebacterium | B_Papillibacter |
| 3    | B_Hungatella | F_Cephalotrichiella* |
| 4    | B_Bacteroides | E_Bacterium* |
| 5    | F_Mycelophthora | B_Corynebacterium |
| 6    | B_DN108009 | B_Bilophila* |
| 7    | B_Intestinalmonas | B_Rikenella* |
| 8    | F_Cystobasidium | B_Lachnospiraceae_NK4B4_group* |
| 9    | F_Metchnikowia | B_Aerococcus* |
| 10   | F_Cutaneotrichosporon | B_Clostridioid* |

*B_" and "F_" represent the microbes belonging to bacteria and fungi, respectively. Rank represents the rank of correlation number.

*Represents the microbes with most correlations found in CP2_LI09 cohort but not CP1_LI09 cohort.
cohorts, some of which were determined to influence the cytokine profiles in the corresponding cohorts. Their biomarker potentials in assisting with the evaluation of better cytokine profiles in the probiotics-treated cohorts deserve further investigation.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA755955 and PRJNA767956.

**ETHICS STATEMENT**

The animal study was reviewed and approved by Animal Care and Use Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

**AUTHOR CONTRIBUTIONS**

HZ and LL designed the study. HZ, QL, JX, SL, and RT conducted the experimental work and prepared the dataset. HZ and KC performed data analyses. HZ and LL wrote the manuscript. All research was conducted under supervision of LL. All authors reviewed the manuscript and approved the submission.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.791152/full#supplementary-material
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