Lactobacillus plantarum LP-Onlly alters the gut flora and attenuates colitis by inducing microbiome alteration in interleukin-10 knockout mice

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Abstract. The association between inflammatory bowel disease (IBD) and gut microbes has been widely investigated. Our previous study demonstrated that *Lactobacillus plantarum* LP-Onlly (LP) applied as a probiotic altered the gut flora and attenuated colitis in interleukin (IL)-10 knockout (IL-$10^{-/-}$) mice. In the present study, metagenome sequencing was performed to investigate the gut microbiome in IL-$10^{-/-}$mice and the influence of oral administration of LP on microbial composition. Metagenomics sequencing was performed to investigate the influence of IBD on the gut microbiome with and without LP treatment. The alteration of the abundances of various taxonomic and functional groups were investigated across these gut microbiomes. The present study demonstrates that *Akkermansia muciniphila* was significantly enriched in IL-$10^{-/-}$ mice, and bacteroides were significantly increased following LP administration. In addition, the phylum *Bacteroidetes* and *Firmicutes* were significantly influenced by LP administration. Further characterization of functional capacity revealed that in the gut metagenomes of IL-$10^{-/-}$mice, genes encoding cell cycle control, replication, recombination, repair and cell envelope biogenesis were decreased, but intracellular trafficking, secretion, and vesicular transport were increased. The present findings indicate that the gut metagenome is associated with IBD, and oral administration of LP contributes to prevention of gut inflammation, providing insight into the treatment of IBD.

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Introduction

The human gut harbors a wide range of microorganisms that determine the hemostasis of the host and enable various metabolic functions, such as biosynthesis of vitamins that humans are not born with (1). The gut microbiota are vital in host health development by interacting with the host (2). A general dysbiosis in microbiota composition and abnormal interactions in gut microbiota may result in various types of disorder. Current gut microbiota studies contribute to the understanding of the complex interactions between biological processes of the microbiota and host. These interactions characterize the underlying mechanism of the association between human health and gut microbiota.

Inflammatory bowel diseases (IBD) is characterized as a group of chronic gastrointestinal inflammation disorders, including Crohn's disease and ulcerative colitis (3). A recent study revealed that IBD is caused by the alterations in gut microbial communities and abnormal interactions between the immune system and the gut microbiota (4). A broad range of microbiota reside in the gastrointestinal tract, including the phylum *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*, which are dominant in the gut microbial community (5). Normally in a healthy gut, *Firmicutes* occupy ~60% of the microbiota, whereas *Bacteroidetes* constitute ~20% of the normal human microbiota (6,7). However, the abundance of microbiota is imbalanced in IBD and the diversity is also reduced (8,9). Microbiota dysbiosis of IBD includes the increased abundance of the phylum *Proteobacteria* and *Bacteroidetes*, while the phyla *Firmicutes* is decreased (10,11). The microbiota composition is associated with gastrointestinal inflammation, therefore the majority of therapeutic strategies for IBD are focused on reconstructing the normal microbiota community of the host gut.

Probiotics are reported to benefit the host, and are non-digestible and fermentable (12). Functional studies of probiotics have been performed in the treatment of a series of inflammatory conditions, including ulcerative colitis and Crohn's disease (13-15). Via stimulating the growth of commensal flora, probiotics alter the composition of the

intestinal microbes and enhance resistance to detrimental bacteria localization, therefore contributing to colitis reduction (16,17). Administration of various probiotic strains has been identified as an effective treatment method for IBD (18). Our previous work demonstrated the administration of *Lactobacillus plantarum* LP-Onlly (LP) may attenuate inflammation of colitis in knockout (IL-10$^{-/-}$) mice (19), however, the underlying mechanism remains unknown.

The aim of the present study was to reveal the alteration of gut microbiota under the influence of LP administration in colitis and clarify the underlying mechanism of LP treatment in experimental colitis. Metagenomic sequencing was performed to investigate the diverse microbiota in IL-10 deficient (IL-10$^{-/-}$) mice with and without LP administration. In addition, a group of wild type (WT) mice and another group of mice with LP treatment (WT + LP) were sequenced to serve as a control. The abundance of microbiota in the LP treated mice (IL-10$^{-/-}$ + LP) and mice without LP treatment (IL-10$^{-/-}$) was compared. *De novo* assembly revealed the taxonomic classification, and further characterized the functional activities of colitis and LP treatment in the gut microbiota of mice.

Materials and methods

Animals. Homozygous IL-10$^{-/-}$ mice (weight, 220±12 g; age, 8 weeks; sex, female) generated on a 129 Sv/Ev background (n=12), and normal 129 Sv/Ev controls (n=12) (The Jackson Laboratory, Bar Harbor, ME, USA) were housed under specific-pathogen-free conditions (temperature, 25˚C; humidity, 70%) in Shanghai Jiao Tong University Medical School (Shanghai, China). Mice were fed a standard sterile diet and filtered water ad libitum under a 12-h light/dark cycle. The animal studies were approved by the Ethical Committee of the Affiliated Sixth People's Hospital of Shanghai Jiao Tong University. Scoring of the disease activity index was performed by an individual blinded to the treatment.

Microbiome genomic DNA extraction and sequencing. Microbiome genomic DNA from mouse stools was prepared using a QIAamp Fast DNA Stool Mini kit (Cat No. 51604, Qiagen GmbH, Hilden, Germany). All samples were sequenced in the Illumina HiSeq2000 instrument at SciLifeLab (Stockholm, Sweden) with up to 10 samples pooled in one lane. Libraries were prepared with a fragment length of 300 bp. Paired-end reads were generated with 100 bp in the forward and reverse direction. Sequencing adapter sequences were removed with cutadapt (http://code.google.com/p/cutadapt/). The length of each read was trimmed using SolexaQA (http://solexaqa.sourceforge.net/) with the options '-b -p 0.05'. Read pairs with either reads <35 bp were removed with a custom Python script. The high-quality reads were then aligned to the human genome (National Center for Biotechnology Information; NCBI version 37) with Bowtie using '-n 2-l 35-e 200-best-p 8-chunkmbs 1024-X 600-tryhard'. This set of high-quality reads was subsequently used for further analysis.

Alignment to reference genomes and taxonomical analysis. A set of 2,797 microbial reference genomes were obtained from the NCBI and Human Microbiome Project (20,21) on 02 August 2011. The reference genomes were combined into two Bowtie indexes and the metagenomic sequence reads were aligned to the reference genomes using Bowtie with parameters '-n 2-l 35-e 200-best-p 8-chunkmbs 1024-X 600-tryhard'. Mapping results were merged by selecting the alignment with fewest mismatches; if a read was aligned to a reference genome with the same number of mismatches, each genome was assigned half to each genome. The relative abundance of each genome was calculated by summing the number of reads aligned to that genome divided by the genome size. In each subject, the relative abundance was scaled to sum to one. The taxonomic rank for every genome was downloaded from NCBI taxonomy to assign each genome to a species, genus and phyla. The relative abundance for each taxonomical rank was calculated by summing the relative abundance of all its members.

Statistical analysis. The high-quality reads were used for *de novo* assembly with Velvet (22) into contigs (length, ≥500 bp) using 3 as the coverage cutoff and a kmer length of 31. To obtain long contigs with high specificity, parameter values for the kmer length and coverage cutoff were iteratively investigated to balance the total assembly length and the N50 value to be used in the final *de novo* assembly. Reads from each subject were used in separate assemblies and unassembled reads were subsequently used in a global final assembly. Genes were predicted on the contigs using MetaGeneMark (23). All genes were then aligned on the contigs with Bowtie using the above-mentioned parameters. The abundance of a predicted gene was calculated by counting the number of reads that align to the gene, and then the read counts were normalized by the gene length and the total mapped reads. The genes were annotated to the eggCOG database (24) with hidden Markov models (HMMs). Protein sequences for microbial orthologs were downloaded and aligned with MUSCLE (25). HMMs were generated with HMMer3 (26) for each KO mouse.

To determine the differential abundance of metagenomic features, an unpaired t-test was applied. Strains and genera with a relative abundance in any subject >10 were included in the analysis. The R package ade4 (27) (https://www.r-project.org/) using instrumental principal component analysis was used to determine the global analysis of species abundance between each group.

The relative abundance of a given taxon in a community was calculated as: Relative abundance = a/b x 100%.

Where 'a' is the number of sequences assigned to the taxon and 'b' is the total number of sequences assigned to all the taxa in the community). Similar calculations were performed for relative abundance of a given gene, Clusters of Orthologous Groups (COG), COG category, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and KEGG subcategory (28). Statistical enrichment of a given gene or COG between two data sets was determined by pairwise comparisons using two-tailed Fisher's exact test, with confidence intervals at 99% significance and Benjamini-Hochberg correction. In all analyses, $P<0.05$ was considered to indicate a significant difference.

Results

LP treatment reduces inflammation in the mouse gut. To investigate the important role of LP in gut microbiota homeostasis,

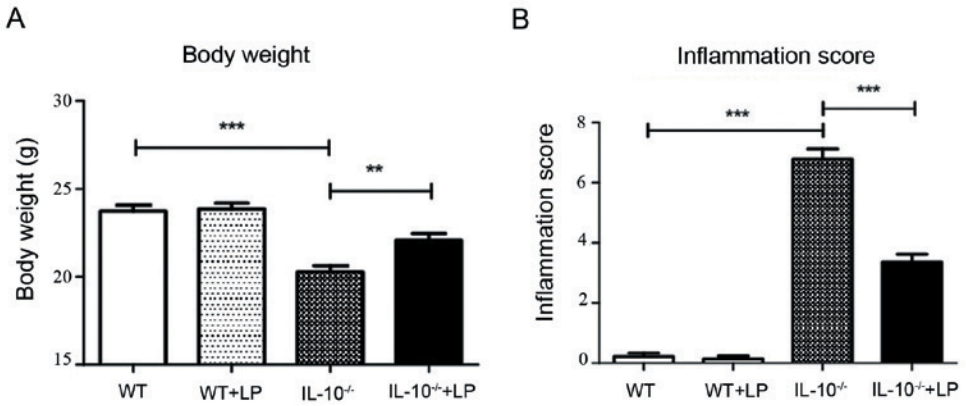

Figure 1. (A) Body weights and (B) inflammation score of IL-10$^{-/-}$ mice and IL-10$^{-/-}$ mice with LP treatment. **P<0.01 and ***P<0.001. IL, interleukin; IL-10$^{-/-}$, IL-10 knockout; WT, wild type; LP, *Lactobacillus plantarum* LP-Onlly.

four groups of mice models were established: WT (WT mice without LP treatment), WT + LP (WT mice with LP treatment), IL-10$^{-/-}$ (IL-10$^{-/-}$ mice without LP treatment), and IL-10$^{-/-}$ + LP (IL-10$^{-/-}$ mice with LP treatment) (n=6/ group). Consistent with a previous study (19), we observed that LP administration attenuated the inflammation syndrome of gut colitis. The body weight of IL-10$^{-/-}$ mice decreased markedly after 4 weeks; however, the body weight of the IL-10$^{-/-}$ mice with LP treatment was significantly greater than that of mice without LP treatment (Fig. 1A). Consistently, the inflammation score of IL-10$^{-/-}$ mice was significantly greater than that of IL-10^{-1-} mice with LP feeding, and no inflammation syndrome was observed in the WT groups (Fig. 1B).

LP administration profoundly affected the gut microbiome. To clarify the influence of LP administration on the gut microbial environment in the gut of colitis mouse models, the gut metagenome of the four groups of mice was sequenced. A total of ~411 million 101-bp paired-end clean reads were generated, the sequencing adapter and low quality reads were removed. The reads from mice genomes were identified and filtered. To reveal the composition of the gut microbiota, all the remaining reads were aligned to a catalog of 2,797 non-redundant NCBI microbial reference genomes (29). On average, 23% of the reads in a sample could be aligned to the reference genome, this ratio was close to the previous metagenome studies (29).

The principal component analysis confirms that the WT samples and IL-10$^{-/-}$ samples were differentiated by the abundance of microbial species. As presented in Fig. 2, samples of control group with LP administration were clustered together and the IL-10$^{-/-}$+ LP samples were clustered in another group. However, the IL-10$^{-/-}$samples without LP administration were dispersed, this may indicate that variation existed among the microbes in the most seriously inflamed gut samples.

To investigate the influence of inflammation on gut microbial communities and regulation of LP administration, the change of identified microbial abundance was compared between the different experiment groups. The species *A. muciniphila* and *Parabacteroides distasonis* were enriched in IL-10$^{-/-}$mice, whereas *Helicobacter hepaticus* was enriched in the control groups (Fig. 3). Previous studies have confirmed the association between these three types of species and IBD.

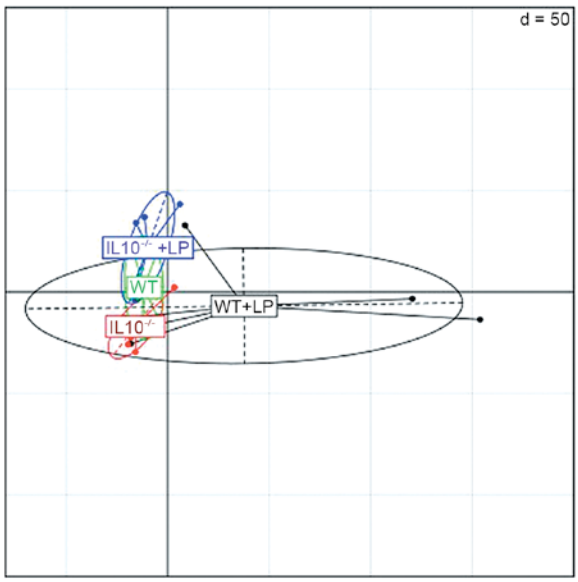

Figure 2. Principal component analysis of microbial species abundance among the four groups of samples. Green is WT mice (n=6), black is WT mice with LP treatment, red is IL-10$^{-/-}$mice (n=6), and blue is IL-10$^{-/-}$mice treated with LP (n=6). WT, wild type; LP, *Lactobacillus plantarum* LP-Onlly; IL, interleukin; IL-10$^{-/-}$, IL-10 knock out.

A. muciniphila exacerbates gut inflammation by disturbing host mucus homeostasis (30). However, the oral administration of *P. distasonis* has been reported as a novel therapeutic strategy for IBD (31). In addition, the *H. hepaticus* was associated with IBD.

L. plantarum is frequently used as a probiotic, and has been associated with the maintenance of intestinal homeostasis and modulation of the immune system. It regulates the quantity of pathogenic bacteria. Following *L. plantarum* administration, the abundance of three types of microbes, namely, *Bacteroides uniformis, P. distasonis* and *Bacteroides salanitronis*, were downregulated compared with the IL-10$^{-/-}$ control groups. *Bacteroides uniformis* have been identified as essential members of gut microbiota, and are enriched in the gut of healthy individuals without IBD (32,33). Furthermore, *P. distasonis* exerts beneficial

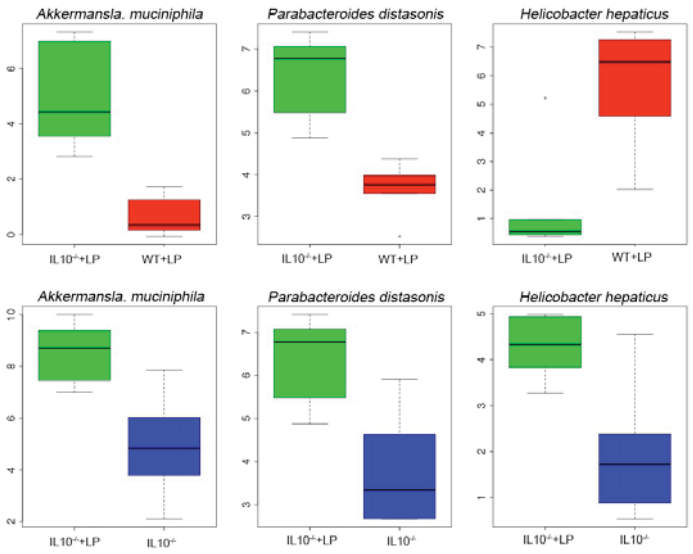

Figure 3. Abundance of genera and species that differ between IL-10$^{-/-}$ and WT and the difference between IL-10$^{-/-}$mice with and without LP treatment. WT, wild type; LP, *Lactobacillus plantarum* LP-Onlly; IL, interleukin; IL-10$^{-/-}$, IL-10 knock out.

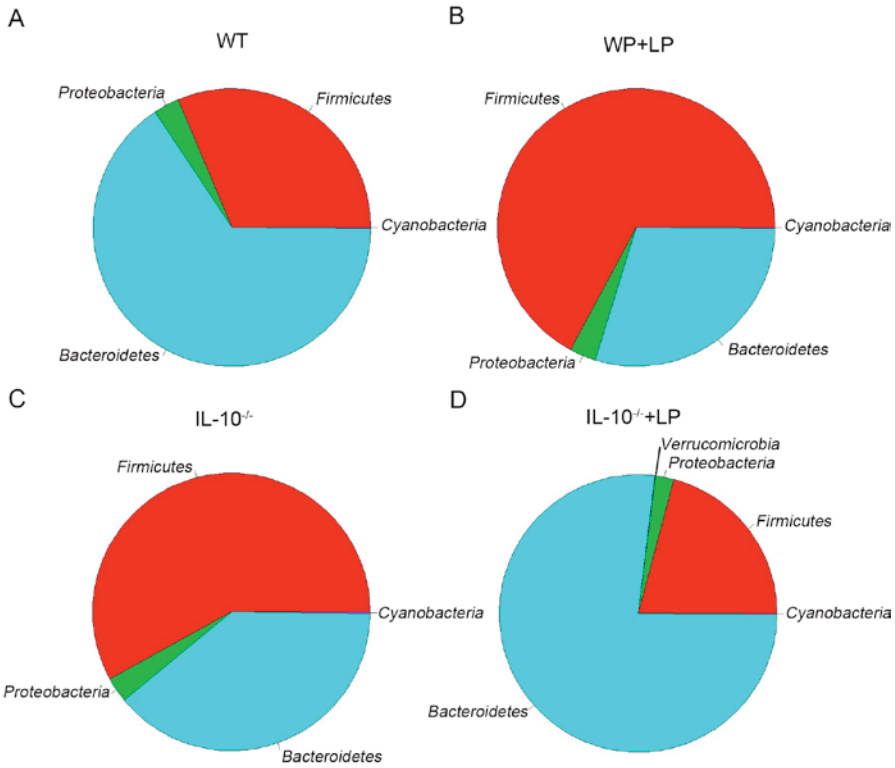

Figure 4. IL-10$^{-/-}$mice and LP treatment affect the proportions of different phyla. The composition of abundant bacterial phyla identified in the gut microbiota of (A) WT mice, (B) WT mice treated with LP, (C) IL-10$^{-/-}$mice (n=7) and (D) IL-10$^{-/-}$mice treated with LP. The undetected phyla are not presented in this figure. WT, wild type; LP, *Lactobacillus plantarum* LP-Onlly; IL, interleukin; IL-10$^{-/-}$, IL-10 knock out.

effects on gut health; Kverka *et al* identified that the oral administration of *P. distasonis* attenuated the inflammation of IBD by modulation of immunity (31). To the best of our knowledge, our study is the first to report the association between *Bacteroides salanitronis* and IBD. These findings may indicate that the loss of probiotic in the IL-10$^{-/-}$ mice gut may aggravate the inflammation and that *L. plantarum* may increase the abundance of probiotic.

Taxonomic characterization in the mice gut microbiomes. To further identify novel genes in the mice gut metagenome and investigate the variation in IL-10$^{-/-}$ mice gut microbiota communities with and without LP administration, the *de novo* assembly was performed for the sequence data. In total 0.32 Gbp of contigs >500 bp were assembled with an N50 value of 0.8 kbp and 5 as the coverage cutoff. Genes were predicted using the assembled contigs and 0.2 million non-redundant

genes were identified. For the phylum level, as presented in Fig. 4, the gut inflammation and LP administration greatly affected the phyla proportion of *Firmicutes* and *Bacteroidetes*, as well as the abundance of various other phyla. This was consistent with previous studies, that the mice gut microbiome was greatly dominated by *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. The ratio of *Bacteroidetes*/*Firmicutes* was markedly decreased in IL-10$^{-/-}$ mice, which may be associated with the inflammation of mice gut; however, following LP administration, the *Bacteroidetes*/*Firmicutes* ratio in IL-10$^{-/-}$ + LP group was increased compared with the IL-10$^{-/-}$ group, and was comparable with the control group. In addition, a small influence on *Cyanobacteria* and *Proteobacteria* was also observed in IBD in the current study. Furthermore, a total of 36 genera/phylum were affected by the inflammation and LP administration. The variation of the abundance of microbiomes in the four groups is presented in Fig. 5.

Functional activities of the mice gut microbiota. Gut microbial activities are associated with host physiological function, may influence metabolism and are a key factor in the inflammation state of IBD. Therefore, to characterize the functional activities of the mice gut microbiota, the predicted genes were functionally annotated to the eggCOG database and the relative abundance of COGs was assessed. The marked variation of COG abundance was observed between the three groups of mice gut microbiotas (Fig. 6). Twenty COG classes demonstrated significant differences in at least two groups of microbiomes. Numerous categories of COG were significantly decreased in the IL-10$^{-/-}$ mice compared with the WT + LP groups, which included 'Cell cycle control, cell division', 'Amino acid transport and metabolism', 'Carbohydrate transport and metabolism', 'Transcription', 'Replication, recombination and repair', 'Cell wall/membrane/envelope biogenesis' and 'General function prediction only', while the abundance of these classes was increased in the LP administration groups of IL-10$^{-/-}$ mice. Furthermore, the abundance of categories 'Intracellular trafficking, secretion and vesicular transport' was significantly enriched in the IL10$^{-/-}$ group.

Discussion

Probiotics have an important role in maintaining gut microbiota homeostasis, the imbalances in microbial communities may contribute to the gut disease of the host (14). Therefore, probiotics have been utilized as an effective treatment for gut disease. The onset and progression of IBD have been attributed to alteration of microbiota composition and the interaction between immune system and microbiota. Our previous work and other studies have demonstrated that probiotic treatment attenuates inflammation of colitis (19). In the current study, a colitis model was constructed using IL-10$^{-/-}$ mice. A metagenome sequence approach was conducted to investigate the effect of LP in colitis in IL-10$^{-/-}$ mice. Weight loss was observed in the IL-10$^{-/-}$mice compared with the control group and the inflammation score was particularly high. However, the inflammation score and weight loss were improved following LP treatment in IL-10$^{-/-}$-deficient mice. This indicated that the colitis mice model had successfully been constructed, and LP administration is effective in the treatment of colitis.

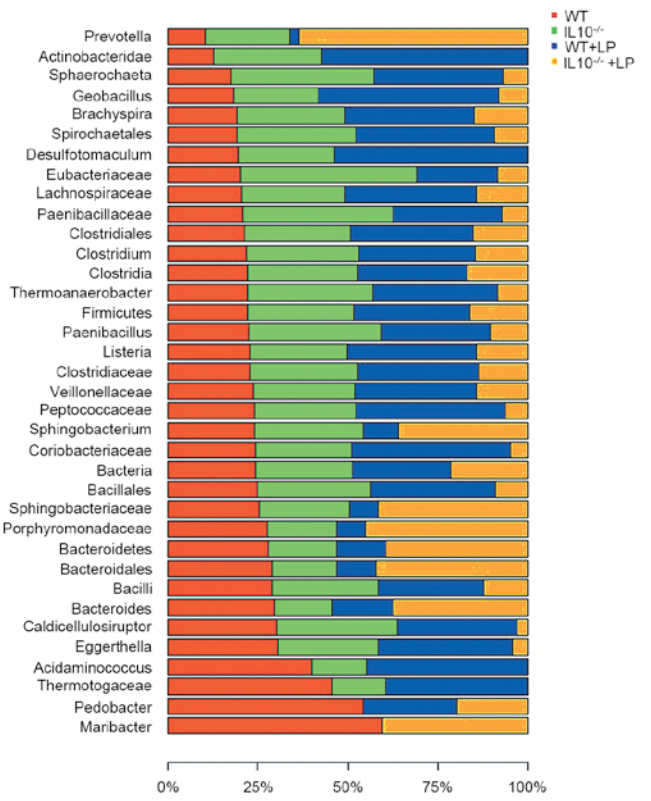

Figure 5. IL-10$^{-/-}$mice and LP treatment affect the proportions of different genera. The composition of these genera significantly affected gut inflammation and were identified in the gut microbiota of IL-10$^{-/-}$mice. Each column is set at 100% to illustrate the proportion of each genus among the different groups; the absence of any color indicates that the genus was not detected in this group of mice. WT, wild type; LP, *Lactobacillus plantarum* LP-Onlly; IL, interleukin; IL-10$^{-/-}$, IL-10 knock out.

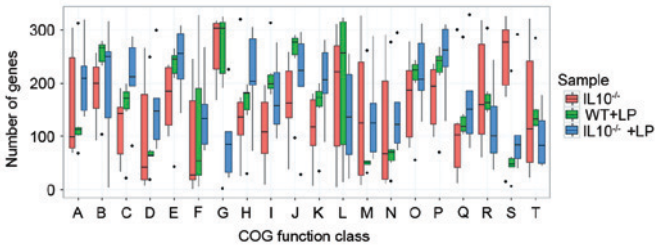

Figure 6. COGs of proteins affected by the IL-10$^{-/-}$ and LP treatments. Occurrence of COGs of proteins according to the following COG functional categories: (A) Energy production and conversion; (B) Cell cycle control cell division; (C) Amino acid transport and metabolism; (D) Nucleotide transport and metabolism; (E) Carbohydrate transport and metabolism; (F) Coenzyme transport and metabolism; (G) Lipid transport and metabolism; (H) Translation, ribosomal structure and biogenesis; (I) Transcription; (J) Replication, recombination and repair; (K) Cell wall/membrane/envelope biogenesis; (L) Cell motility; (M) Posttranslational modification, protein turnover, chaperones; (N) Inorganic ion transport and metabolism; (O) Secondary metabolites biosynthesis, transport and catabolism; (P) General function prediction only; (Q) Function unknown; (R) Signal transduction mechanisms; (S) Intracellular trafficking, secretion and vesicular transport; (T) Defense mechanisms. COGs, Clusters of Orthologous Groups; WT, wild type; LP, *Lactobacillus plantarum* LP-Onlly; IL, interleukin; IL-10$^{-/-}$, IL-10 knock out.

During the metagenome analysis, clean reads were mapped to microbial reference genomes. The principal component analysis clearly differentiated the IL10$^{-/-}$ + LP group and WT + LP group; however, the samples of IL-10$^{-/-}$ are scattered.

This may be because inflammation of colitis influences the abundance and diversity of microbiota. The abundance of *A. muciniphila* and *P. distasonis* was significantly increased in IL-$10^{-/-}$ + LP group compared with the WT + LP group. The abundance of *B. uniformis* and *P. distasonis* was decreased in the IL-$10^{-/-}$ group. A previous study revealed that the increase of *A. muciniphila* was harmful to IBD and that *B. uniformis* has been identified as an essential member of gut microbiota (34). However, a previous study demonstrated that *P. distasonis* may be beneficial to IBD as it prevents intestinal inflammation in murine models (31). In addition, taxonomic analysis revealed that *Firmicutes* and *Bacteroidetes* are dominated in mice guts, which is consistent with previous studies. The *Firmicutes*/*Bacteroidetes* ratio increases in IL-$10^{-/-}$, and following LP administration this ratio decreased to a normal level when compared with the ratio of WT. However, this ratio increased markedly in the WT + LP group. The results demonstrate that alterations to the microbiota composition may serve an important role in IBD, and the administration of LP may regulate the abundance and diversity of gut microbiota.

In the analysis of functional activities, the relative abundance of COGs was observed to change in IL-$10^{-/-}$mice. The function categories of COG, including 'Cell cycle control, cell division', 'Amino acid transport and metabolism', 'Carbohydrate transport and metabolism', 'Transcription' and 'Replication, recombination and repair' were significantly influenced. The 'Carbohydrate and nucleotide metabolism', 'Lipid and amino acid metabolism' and 'Amino acid transport and metabolism' were found to be associated with IBD in a recent study (5).

In conclusion, the present study further demonstrated the effectiveness of LP in the treatment of colitis. The current study provided an overview of gut microbiota components of colitis, and revealed the ability of LP to regulate the gut microbiota, which may be important in attenuating the inflammation of colitis. However, further investigations regarding LP and gut microbiota in colitis are required to reveal how metabolic changes to LP attenuates the colitis, and how the host immune system interacts with LP.

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