Lactiplantibacillus plantarum P9 alleviates chronic diarrhea via modulation of gut microbiota and its intestinal metabolites: a double-blind, randomized, placebo-controlled study

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Article

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Abstract

This study evaluated the beneficial effects of administering *Lactiplantibacillus plantarum* P9 (P9) on chronic diarrhea. A randomized, double-blind, placebo-controlled trial was performed. Patients were assigned to the probiotic or placebo group randomly. The primary endpoint was the diarrhea symptom severity score; the secondary endpoints were the stool consistency, the number of bowel movements, fecal urgency score, the Depression Anxiety Stress Scales-21 score, fecal metagenome and metabolome. Administering P9 for 4-week significantly improved diarrhea symptoms and the stool consistency, accompanied by a multitude of patients' gut microbiota and metabolome changes: increases in several gut short-chain fatty acid (SCFA)-producers and a bile acid metabolizing species; elevation in fecal metabolites of bile acids, amino acids, and short-chain fatty acids; increases in cumulative gene abundances of 15 carbohydrate-active enzyme subfamilies; increases in fecal acetate and butyrate concentrations. P9 administration had a remarkable therapeutic effect on chronic diarrhea, supporting using probiotics to alleviate chronic diarrhea.

Introduction

Chronic diarrhea is one of the most common functional gastrointestinal (GI) diseases and is defined as more than three bowel movements or loose stools per day for at least four weeks[1]. Its prevalence worldwide is estimated to be 3–20%[2]. Generally, the most common causes of chronic diarrhea are functional diseases, such as irritable bowel syndrome (IBS) with diarrhea (IBS-D) and functional diarrhea, resulting in a multitude of clinical and social problems for the patients[3]. Currently available drugs for managing chronic diarrhea include loperamide, diphenoxylate, clonidine, codeine, alosetron, morphine preparations, anti-depressants, and anticholinergic medications[4]. However, these drug treatments cause several common GI side effects, like constipation, nausea, vomiting, bloating, and diarrhea[5, 6]. A fairly recent study also reported that antidepressants induces antibiotic resistance and persistence[7]. Therefore, it is particularly challenging to explore novel methods for managing chronic diarrhea. Growing evidence supports that gut dysbiosis increases the susceptibility to various pathogens, and, in diarrhea is both a cause and consequence of the reduction in gut microbial diversity and dysbiosis[8, 9]. In host-microbe interaction, lots of microbial metabolites are produced, including bile acids, short-chain fatty acids (SCFAs), and amino acids; many of these metabolites are involved in GI inflammation and colonic homeostasis[10, 11]. In addition, more studies have now focused in dissecting the role of gut phageome in health, as phages are able to modify the gut microbiota. Phages may also play a part in GI diseases, such as inflammatory bowel disease (IBD) and colorectal cancer. Moreover, the gut phageome profile showed significant alterations in diarrheal patients[12, 13]. Therefore, the gut microbiota, metabolome, and phageome can be considered targets for treatment of diarrhea.

Probiotics are defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host"[14]. Probiotics have been commonly applied in managing diarrhea, and it has shown obvious symptom alleviation effect, accompanied by the reduction in gut inflammation,

restoration of gut microbiota balance and the intestinal mucosal barrier function[15]. The species, *Saccharomyces boulardii*, was reported to relieve diarrhea symptoms by modulating the gut microbiota that inhibit gut motility in mice[16]. A multispecies probiotic mixture (containing *Lactobacillus acidophilus* S5, *Bacillus subtilis* no. Bzg988118, and *Saccharomyces cerevisiae* SHZ2017) could reduce the incidence of diarrhea, increase the growth performance, and balance the fecal microbiota in pre-weaning calves[17]. Unlike animal studies, clinical trials have produced controversial results regarding diarrhea. For example, one study showed that administering oral *L. plantarum* CCFM1143 for four weeks could substantially improve defecation frequency, stool consistency, health status, and quality of life of patients with chronic diarrhea[18]. Another randomized, double-blind, placebo-controlled study found that after 8-week probiotic intervention with a multi-strain mixture of *Bifidobacterium, Lactobacillus*, and *Streptococcus thermophilus*, the total IBS-severity scoring system score reduced obviously in patients with diarrhea, although no significant differences were seen in the mean number of bowel movements, stool consistency, and fecal urgency between the probiotic and placebo groups[19]. Similarly, two months of *L. plantarum* CJLP243 administration substantially increased the proportion of patients of adequate functional diarrhea relief, with reduced loose stool frequency[20]. However, 8-week intervention probiotic fermented milk (containing *L. paracasei* F19, *L. acidophilus* La5 and *B. lactis* Bb12) exerted no obvious clinical effect on GI symptoms, quality of life, and psychological symptoms in IBS patients[21]. In general, the beneficial effects of probiotics on diarrhea are related to the strain, dosage, sample size, and duration in clinical trials. Thus, extensive and rigorous clinical studies and reproducible microbiological analysis are required to elucidate the role and functional effects of probiotics in managing chronic diarrhea.

Lactobacillus plantarum P9 (P9) is a potential probiotic strain isolated from traditional acidic gruel[22]; the strain has high tolerance to gastroenteric fluid, strong immune-enhancing, anti-inflammatory, and intestinal immune modulatory activities[23, 24]. The latest research reported that consuming P9 could alleviate constipation symptoms in patients with chronic constipation, and the symptom alleviation was accompanied by the increases in functional intestinal microbiota (*L. plantarum*, *Oscillospiraceae sp.*, and *Lachnospiraceae sp.*) and microbial metabolites (L-asparagine, L-pipecolinic acid, valeric acid and caprylic acid)[25]. The aim of the current four-week randomized, double-blind, placebo-controlled study was to evaluate the symptom alleviation effect and safety of P9 in managing chronic diarrhea. The primary endpoint was the diarrhea symptom severity score; the secondary endpoints were the stool consistency, frequency, and urgency, and gut microbiota, phageome, and metabolite biomarkers. We demonstrated that P9 consumption could improve diarrhea, and the symptom relief was interrelated with desired changes in the functional gut microbiota, phageome, and microbial metabolome. This study supports that probiotics such as P9 can be used for managing chronic diarrhea; and its beneficial mechanism was explored from the perspectives of gut microbiota and metabolome.

Results

Demographic data

A total of 189 patients were randomized to receive P9 or placebo. After a 4-week intervention period, eleven and eight patients from the placebo and P9 group were excluded because of decline participation or missing diary (Fig. 1a; Figure S1). Thus, 170 patients (85 supplemented P9 and 85 receiving placebo) completed the study. Patients were aged 22.35 ± 4.07 years and 22.11 ± 4.90 years in P9 and placebo groups, respectively. The proportion of male subjects in the P9 and placebo groups was 47.06% and 50.59%, and that of female subjects was 52.94% and 49.41%, respectively. The ratio of Han ethnic to other ethnic groups was 82 to 3 and 81 to 4 in P9 and placebo groups, respectively (Table S2). There was no significant difference between the P9 and placebo groups in terms of the baseline age, gender ratio, ethnicity, history of drug allergy, preexisting disease, and previous drug treatment ($P > 0.25$ in all cases; Table S2).

P9 administration apparently improved diarrhea symptoms

The diarrhea symptom severity score was based on the Gastrointestinal Symptom Rating Scale. Before the intervention, no significant difference existed in the diarrhea symptom severity score between the P9 and placebo groups (2.013 ± 0.977 and 2.074 ± 0.874 in P9 and placebo groups, respectively, $P = 0.588$; Fig. 1b; Table S3). After the 28-day intervention, the diarrhea symptom severity score in P9 group was significantly lower than the placebo group, reduced by 20.6% (1.138 ± 0.694 and 1.433 ± 0.803 in P9 and placebo groups, respectively, $P = 0.048$; Fig. 1b), indicating that P9 intervention can relieve diarrhea symptoms.

At baseline (day 0), no significant differences were observed in all secondary endpoint parameters, including the scores of bowel frequency, stool consistency, fecal urgency, and DASS-21 questionnaire were noted between P9 and the placebo groups ($P > 0.05$; Fig. 1b; Table S3). However, compared with the placebo group, after the 28-day intervention, patients in the P9 group had statistically significant improvement in stool consistency score (4.717 ± 0.725 and 4.933 ± 0.626 in P9 and placebo groups, respectively; $P < 0.05$; Fig. 1b), though not the scores of bowel frequency, fecal urgency, and the depression, anxiety and stress levels ($P > 0.05$; Figure S1b).

Although no significant difference was observed in the number of adverse events between P9 and the placebo groups ($P = 0.218$; Table S3), obviously fewer adverse events were reported by the patients in P9 group than those in the placebo group at day 28 (six vs 14 events) and day 42 (three vs five events). No serious adverse reactions were reported in any of the groups at any time point.

P9 supplementation modulated patients' fecal bacterial microbiota diversity and composition

The fecal microbiota of 169 patients was analyzed at three time points (days 0, 28, and 42) by metagenomic sequencing. One patient from P9 group did not provide stool sample for fecal metagenome analysis (Fig. 1a).

No significant differences were observed in the alpha diversity (Shannon and Simpson indices, $P > 0.05$) between the P9 and placebo groups at any time point (Fig. 2a). However, beta diversity analyzed by PCoA and adonis test revealed significant differences in the fecal microbiota structure between P9 and placebo groups at days 28 ($R^2 = 0.015$, $P = 0.012$) and 42 ($R^2 = 0.014$, $P = 0.018$; Fig. 2b).

We also found that P9 administration was associated with post-intervention changes in the species-level fecal microbiota composition. A total of 29 major SGBs (mainly belonging to *Lactobacillaceae*, *Oscillospiraceae*, *Ruminococcaceae, Lachnospira*) were identified; none of them showed significant differences between the two groups at baseline (day 0), and they only became differentially abundant at days 28 and 42 (19 and 10 SGBs, respectively; Fig. 2c; Table S4). After 28-day intervention, the fecal microbiota of P9 group had significantly more *Lactiplantibacillus plantarum* and *Ruminococcus_*A *faecicola* compared with the placebo group, while an opposite trend was observed in the species, *Mediterraneibacter torques*, *Eubacterium_*I *ramulus*, and *Enterocloster* sp000431375 ($P < 0.05$ in all cases; Fig. 2c). At day 42, significantly more *Butyricicoccus_*A sp002395695 and *Streptococcus thermophilus* were detected in P9 group compared with the placebo group, and *Phascolarctobacterium faecium* and *Faecalibacterium* sp. showed an opposite trend ($P < 0.05$ in all cases; Fig. 2c). Interestingly, eight SGBs showed consistent differences at days 28 and 42, including significantly more *Acutalibacteraceae* sp000431775 and *Paraprevotella xylaniphila*, but significantly fewer *Coprococcus* sp and *Butyricimonas virosa* in P9 group compared with the placebo group ($P < 0.05$ in all cases; Fig. 2c). Taken together, these results suggested that P9 supplementation could significantly change the diversity and composition of gut microbiota in patients with chronic diarrhea.

We further analyzed the correlation between the species-level gut microbiota and clinical indicators of diarrhea to investigate whether the improvement of clinical indicators was related to changes in specific bacteria after P9 administration (Fig. 2d). Our results showed that the stool consistency score correlated positively with *Eubacterium_*I *ramulus* ($r = 0.20$, $P = 0.019$); the stress score correlated positively with *Enterocloster* sp000431375 ($r = 0.21$, $P = 0.008$); and the bowel frequency showed a significant positive correlation with *Mediterraneibacter torques* ($r = 0.24$, $P = 0.002$). Moreover, the anxiety score showed a significant negative correlation with *Ruminococcus_*A *faecicola* ($r = -0.20$, $P = 0.019$), suggesting that the symptom alleviation was associated with changes in some specific functional gut bacteria.

P9 supplementation modulated patients' fecal phageome diversity and structure

Since phages play a part in modifying the gut microbiota, we then investigated intervention-associated changes in patients' fecal phageome diversity and composition. A total of 94,384 nonredundant vOTUs were annotated by comparing our dataset against the Metagenomic Gut Virus catalogue; and 41,059 vOTUs were assigned into 13 bacteriophage families, including 7,587 prophages and 33,472 non-prophages (Fig. 3a). Taxonomic annotations of these sequences found a high prevalence of *Siphoviridae*

(33.15%), *Myoviridae* (9.31%), *Microviridae* (5.07%), *Podoviridae* (1.91%), and *crAss-phage* (1.03%; Fig. 3a), most of which belonged to the order Caudovirales (87.67%; Fig. 3a).

Beta diversity analysis showed that no significant differences were found in the overall phageome between P9 and the placebo groups at days 0, 28, and 42 (Fig. 3b). Consistently, there were no significant differences in the alpha diversity of phageome between the two groups at days 0 and 42 ($P > 0.05$; Fig. 3c), except that, at day 28, the Simpson index of patients' fecal phageome in P9 group was numerically lower than that of the placebo group ($P = 0.056$; Fig. 3c). Interestingly, we observed a significant positive correlation between the Shannon index of the gut bacteria microbiota and phageome ($R = 0.928$, $P < 0.001$; Fig. 3d), which was consistent with the results of Procrustes analysis ($R = 0.818$, $P = 0.001$; Fig. 3e), suggesting that there was a strong cooperativeness between the gut phageome and their bacterial hosts.

Phages are obligate intracellular parasites residing in bacterial host, so specific genomic associations between phages and bacteria reflect the phage infection history. To further explore the interplay between gut bacteriophages and bacteria, we then investigated specific distribution of phage sequence in bacterial host genomes (Fig. 3f). A total of 21,103 vOTUs annotated to 12 known bacteriophage families were analyzed; 94.2% of these vOTUs (corresponding to 19,874 vOTUs) were predicted to connect with specific bacteria hosts, and 12,870 of them were connected to known host bacterial genera. *Siphoviridae*, the most highly widespread gut phage family, were mainly associated with Firmicutes and Bacteroidota hosts (including the genera *Ruminococcus*, *Bacteroides*, *Faecalibacterium*, *Eubacterium*, and *Lachnospira*). *Myoviridae* and *Microviridae* are two widespread and abundant human gut phage families often infected Firmicutes and Bacteroidota hosts (including the genera *Faecalibacterium*, *Lachnospira*, and *Bacteroides*). In addition, *crAss-phage* mainly infected Bacteroides hosts (*Bacteroides* and *Prevotella*), and *Herelleviridae* mainly infected Firmicutes hosts (*Flavonifractor*). Surprisingly, most of these infected bacterial hosts, including *Faecalibacterium*, *Eubacterium 28*, and *Lachnospira*, changed significantly after P9 intervention.

Finally, we explored the association between patients' gut bacteriophages and clinical indicators after P9 intervention. A correlation analysis of clinical indicators and 12 known bacteriophage families was performed (Fig. 3g). Our results showed that both *Microviridae* ($r = 0.20$, $P = 0.012$) and *crAss-phage* ($r = 0.23$, $P = 0.003$) were positively correlated with the fecal urgency score; and *Herelleviridae* showed a positive correlation with bowel frequency ($r = 0.20$, $P = 0.019$). These results suggested changes in specific phage sequences after P9 supplementation were associated with diarrhea improvement.

P9 supplementation modulated patients' gut bioactive metabolites and CAZymes

A genome-centric metabolic reconstruction was established to identify intervention-associated changes in GMMs encoded in 629 SGBs using the MetaCyc and Kyoto Encyclopedia of Genes and Genomes databases. A total of 72 GMMs were identified, belonging to 11 metabolic modules, including SCFAs,

amino acids, tryptophan and its derivatives, unsaturated fatty acids, monosaccharides, disaccharides, polysaccharides, neurotransmitters, vitamins, bile acids and other metabolic modules (Fig. 4a). These identified modules were encoded by 34 bacterial orders. The modules of acetate synthesis, quinolinic acid degradation, and S-adenosylmethionine synthesis were common to most orders (Fig. 4a). However, some orders exhibited a higher metabolic diversity than other orders. The top three metabolically diverse orders were *Bacteroidales*, followed by *Lachnospirales* and *Oscillospirales* (belonging to Firmicutes), encoding a wide array of metabolic modules related to SCFA, amino acid, vitamin, and bile acid metabolism. Interestingly, several bacteria belonged to these three orders (including *Lactiplantibacillus plantarum*, *Paraprevotella xylaniphila*, and *Acutalibacteraceae* sp000431775) showed significant differential abundance after P9 intervention. Modulation of the composition of these metabolic diverse taxa may drastically change the potential metabolic function of the gut microbiota.

We then analyzed changes of potential gut bioactive metabolites after P9 intervention using the MelonnPan pipeline, and we found no significant difference in the alpha diversity of gut bioactive metabolite profile between the P9 and placebo groups at days 0 and 42, but the Shannon index in the P9 group was almost significantly higher than that of the placebo group at day 28 ($P = 0.059$; Fig. 4b). The results of alpha diversity analysis were in line with that of the beta diversity analysis by PCoA and adonis test. Significant differences were observed in the gut bioactive metabolites profile between the P9 and placebo groups at day 28 ($R^2 = 0.028$, $P = 0.013$), but not at day 0 ($R^2 = 0.004$, $P = 0.538$) and 42 ($R^2 = 0.008$, $P = 0.069$; Fig. 4c). Moreover, a total of 18 differentially gut active metabolites between the P9 and placebo groups were probiotic intervention-specific; these predicted metabolites only became significantly different between groups after 28-day P9 intervention but not at baseline (Fig. 4d, Table S5). Several of these probiotic-intervention responsive metabolites, such as cholate, chenodeoxycholate, C2 carnitine, C16 carnitine, cholesterol, X2 hydroxyphenethylamine, creatine, and bilirubin, were significantly enriched in P9 group compared with the placebo group ($P < 0.05$; Fig. 4d). To further explore the enzyme repertoire for complex polysaccharide metabolism encoded by the patients' fecal microbiota, CAZyme genes were annotated using dbCAN2. A total of 26,170 CAZyme-encoding genes were found across 629 SGBs (Table S6), and most genes encoded the family glycoside hydrolases (GHs, 14,598 genes), followed by glycosyltransferases (GTs, 6,108 genes), carbohydrate esterases (CEs, 2,726 genes), carbohydrate-binding modules (CBMs, 1,690 genes), polysaccharide lyases (PL, 810 genes), and auxiliary activities (AAs, 238 genes). In addition, comparative analysis of CAZyme-encoding subfamilies between P9 and placebo groups found that 15 CAZyme subfamilies were enriched in P9 group, including: glycoside hydrolases (GH108, GH13_13, GH13_2, GH13_27, GH158, GH64, GH5_18), carbohydrate-binding modules (CBM4, CBM56), glycosyltransferases (GT21, GT60, GT74), polysaccharide lyases (PL1_3, PL10_2), and CE16 (Fig. 4e). These results together indicated that P9 administration could enrich the CAZyme-encoding genes in the gut microbiota, possibly contributing to a broadened carbohydrate utilization capacity.

P9 supplementation modulated patients' fecal metabolome

Next, we analyzed specific changes in the fecal metabolome of the patients after P9 intervention. On the fecal metabolome PCA plot, symbols representing the quality control samples formed a close cluster (Fig. 5a), indicating a good stability of the instrumental conditions and the reliability of analysis of other samples. The PLS-DA analysis of the fecal metabolomes of samples from the two groups showed that there was a moderate degree of group-based separation at all three time points, although no significant difference was detected by adonis test ($P > 0.05$; Fig. 5b). We further analyzed the metabolite-level differences in the fecal metabolomes between two groups based on the VIP value generated by the PLS-DA model (VIP > 2.0) and the *P* value calculated by Wilcoxon test ($P < 0.05$). A total of 21 significant differential metabolites were identified at days 28 and 42 (Fig. 5c; Table S7). These metabolites were not significantly different between the two groups at baseline. Specifically, compared with the placebo group, P9 group had significantly more caffeic acid, taurine, 3-hydroxypentanoic acid, hexacosanoic acid, and cerotic acid after intervention ($P < 0.05$; Figure. 5d). We further explored the associations between differentially abundant metabolites and clinical indicators of diarrhea by performing Pearson correlation analysis. Interestingly, the results showed that caffeic acid was significantly and negatively correlated with the depression score ($r = 0.21$, $P < 0.01$); and taurine was significantly and negatively correlated with the diarrhea severity score ($r = 0.21$, $P < 0.01$; Figure. 5e).

Afterwards, we analyzed the SCFA composition in patients' feces by GC-MS to identify differential abundant SCFAs associated with P9 intervention. After 28 days of intervention, more acetic acid and butyric acid were detected in P9 group compared with the placebo group ($P < 0.05$; Fig. 6a), but the fecal contents of isovaleric acid, valeric acid, propionic acid, and isobutyric acid exhibited no significant differences between groups ($P > 0.05$; Fig. 6a). These results confirmed that P9 intervention could increase patients' functional gut metabolites like acetic acid and butyric acid.

Discussion

Chronic diarrhea is a multifactorial disorder that causes significant psychological distress to patients and economic burden on the health care system[26]. Probiotics are increasingly prescribed for managing chronic diarrhea. However, previous intervention studies of probiotic-based diarrhea management mostly focused on reporting the clinical outcome[27, 28, 29], without clarifying the role of host gut microbiota in the diarrhea relief effect using systematic multi-omics. Therefore, here we conducted a randomized, double-blind, placebo-controlled study involving 170 subjects to explore the beneficial effect of administrating P9 in alleviating chronic diarrhea. Our results showed that P9 intervention could effectively relieve both the severity of diarrhea symptom and stool consistency. In addition, by integrating multiple omics strategies, we demonstrated that certain gut bacteria (*Lactiplantibacillus plantarum*, *Ruminococcus_A faecicola*, and *Paraprevotella xylaniphila*), bacteriophages (*Herelleviridae* and *Microviridae*), and microbial metabolites (cholate, chenodeoxycholate, acetic acid, and butyric acid) may contribute to diarrhea relief symptoms through function-driven changes and co-interactions in the treatment process (Fig. 6b).

Our first finding is that P9 administration significantly improved patients' diarrhea symptom, evident by the significant decreases in diarrheal severity score and stool consistency of the probiotic recipients at

day 28 compared with subjects receiving placebo. Some previous studies also observed that probiotic application could relieve chronic diarrhea. For example, a previous study found that a 4-week treatment of *L. plantarum* CCFM1143 decreased the defecation frequency and Bristol score in patients with chronic diarrhea[18]. Another placebo-controlled study reported that a 2-month *L. plantarum* CJLP243 treatment reduced loose stool frequency[20]. However, our study found that administering P9 treatment did not improve the bowel movement frequency/urgency and DASS-21 scores, which is consistent with the results reported by Bai et al. (2023)[30]. Meanwhile, no serious adverse events occurred in any of the patients during the intervention period. Thus, the use of P9 as a dietary intervention is a safe and effective alternative strategy to relieve chronic diarrhea.

Improving the balance of the gut microbiota and inhibiting the colonization of harmful bacteria are known mechanisms of probiotics in alleviating diarrhea[31]. Thus, we analyzed the changes in the host gut microbiota in the course of P9 intervention. Our study found no significant changes in the alpha diversity (Shannon and Simpson diversity indexes) of the intestinal bacterial and bacteriophage microbiota in patients. However, interestingly, PCoA analysis revealed that the gut microbiota structure of subjects in P9 group was significantly modulated after 28-day intervention. We further looked into our dataset to identify P9-responsive gut bacteria and bacteriophages, revealing that P9 administration could substantially enrich taxa like *Acutalibacteraceae* sp000431775, *Paraprevotella xylaniphila*, *Ruminococcus*_A *faecicola*, *Butyricicoccus*_A sp002395695, and *Streptococcus thermophilus* in subjects' gut microbiota, while significantly decreasing the levels of *Coprococcus* sp., *Mediterraneibacter torques*, and *Enterocloster* sp000431375. Members of *Acutalibacteraceae* are involved in bile acid metabolism, improving intestinal stability through bile tolerance and degradation of host mucous polysaccharides[32]. *Paraprevotella xylaniphila*, *Ruminococcus*_A *faecicola*, and *Butyricicoccus*_A sp002395695 are SCFA producers, especially butyric acid and acetic acid. These SCFAs are significantly reduced in patients with IBD and antibiotic-associated diarrhea[33, 34, 35, 36]. *Mediterraneibacter torques*, also known as *Ruminococcus torques*, was positively correlated with intestinal permeability and GI diseases, and it was found to be enriched in the gut microbiota of patients with IBS-D[37, 38]. We also found that bowel frequency was significantly and positively correlated with *Mediterraneibacter torques* and the viral family of *Herelleviridae*. More *Coprococcus* have been reported in antibiotic-associated diarrhea mice and patients with Crohn's disease[39, 40]. Notably, most of the aforementioned undesired bacteria belong to the phyla Firmicutes and Bacteroidetes, which are commonly infected by the phage families *Siphoviridae*, *Microviridae*, *crAss-phage*, and *Herelleviridae*, raising the idea of implementing phage therapy for tackling enteric diseases[13].

In addition, our results showed that *crAss-phage* and *Microviridae* had significant positive correlation with fecal urgency score. It has been reported that patients with GI diseases (such as ulcerative colitis[41], IBS-D[42], and colorectal cancer[43]) had significantly more *crAss-phage*, *Microviridae*, and *Herelleviridae* than healthy subjects, suggesting high levels of gut *crAss-phage*, *Microviridae* and *Herelleviridae* sequences might be linked to diarrhea. These results suggested that desirable changes and interactions between gut

bacteria and bacteriophages in patients could be a potential explanation for the effectiveness of P9 in relieving diarrhea.

The gut microbiota metabolizes dietary components and nutrients to produce functional metabolites, modulating the host metabolic processes[44]. Our results showed that bile acids, amino acids, and SCFAs metabolic modules were enriched in most of fecal SGBs after P9 administration. Malabsorption or overproduction of bile acids could lead to chronic watery diarrhea[45]. Sinha et al.[46] reported that secondary bile acids deficiency promoted gut inflammation in patients with ulcerative colitis, and restoring the level of secondary bile acids or secondary bile acid-producing bacteria (such *Ruminococcaceae*) could reduce intestinal inflammation. Consistently, we observed a significant increase in the predicted levels of cholate and chenodeoxycholate in the fecal microbiota of patients in P9 compared with those in the placebo group after P9 intervention. Cholate has been reported to protect the intestinal epithelial barrier and regulate the intestinal mucosal immune function[47]. As one of the main secondary bile acids, chenodeoxycholic acid has been found to accelerate colonic transit and decrease stool consistency in healthy volunteers[48], and the oral intake of chenodeoxycholic acid could alleviate diarrhea symptoms in patients with cerebrotendinous xanthomatosis[49]. Our results suggested P9 administration could in the fecal microbiota-encoded potential of cholate and chenodeoxycholic acid production, which might have played a role in improving intestinal barrier homeostasis and reduce intestinal inflammation in diarrheal patients.

Another interesting result of study is the observations of changes in the predicted levels of glutamate and taurine. The predicted level of glutamate decreased in the gut microbiome of P9 group after probiotic intervention. Glutamate affects the survival of gut microbiota and participates in shaping the gut ecological environment and may be unfavorable to host health. Dietary glutamate has been found to aggravate the symptoms of IBS, increasing the intestinal dysmotility and sensitivity of pain in patients with IBS-D[50, 51]. Moreover, the predicted level of taurine in the fecal metabolome of patients receiving P9 significantly increased. Previous animal studies have shown that taurine could increase intestinal epithelial barrier integrity via enhancing the expression of tight junction proteins[52]. Taurine supplementation was found to significantly attenuate the severity of diarrhea[53]. Concordantly, our study found a significant negative correlation between taurine and diarrheal severity score, suggesting that regulation of amino acid metabolism could be a possible pathway of P9 in alleviating. Additionally, there were significantly increases in the levels of other predicted gut bioactive metabolites (such as carnitine and bilirubin) and fecal metabolites (such as caffeic acid, and long-chain fatty acids like hexacosanoic acid and cerotic acid) after P9 intervention. In vitro and animal studies have shown that both carnitine and bilirubin could lessen colonic oxidative stress and inflammation, thereby reducing the severity of colitis[54, 55]. Thus, a high intestinal carnitine and bilirubin levels could be part of the mechanism of mitigation of diarrhea after P9 administration. Moreover, our correlation analysis found that caffeic acid associated significantly and negatively with the depression score. Caffeic acid supplementation has been shown to be able to reverse colitis-related gut dysbiosis, gut barrier damage, and infiltration of inflammatory cells in colonic tissues in colitis mice[56]. A previous study found that a caffeic acid-

containing plant in a Mexican traditional medicine was effective in treating diarrhea and dysentery[57]. A previous animal study found that bacterial long-chain fatty acids could alleviate colitis and decrease inflammation in mice by activating the peroxisome proliferator-activated receptor gamma[58].

The human intestinal microbiota genome encodes a large number of CAZYmes, through which bacteria hydrolyze a wide array of fibers to produce functional metabolites[59]. Thus, the profile of CAZYmes encoded by the gut microbiota reflects its carbohydrate metabolic capacity. Another important and interesting finding in this study was the identification of a panel of differentially abundant metabolic modules between P9 and the placebo groups after the intervention, including 15 significant increased subfamilies of CAZymes, most of which were GHs, GTs, PLs and CBMs. These are key enzymes for decomposing complex carbohydrates, generating SCFAs and other metabolic by-products that promote host energy acquisition[60, 61]. By GC-MS analysis, we found that the fecal acetic acid and butyric acid concentrations were significantly higher in P9 group compared with the placebo group. Consistently, a previous study found that *L. plantarum* CCFM1143 administration significantly raised the levels of acetic acid and propionic acid in diarrhea patients compared to the placebo group[18]. Short-chain fatty acids, including acetate, butyrate and propionate, are some of the major metabolites of gut microbiota responsible for bridging the microbial-host interaction[62, 63]. Oral acetate administration significantly alleviated colitis in mice by improving intestinal permeability[64]. Butyric acid is a key energy source for enterocytes, and it exerts anti-inflammatory effect and protective effect on the intestinal epithelial barrier[65, 66].We also confirmed that the fecal content of 3-hydroxyvaleric acid was significantly higher in P9 group compared to placebo group. 3-hydroxyvaleric acid is a SCFA that is biochemically related to valeric acid, but its function requires further investigation. Overall, the significant increase in intestinal SCFAs, particularly acetic acid and butyric acid, subsequent to P9 intervention could play an important role in promoting the repair of intestinal barrier and intestinal immune function, thereby alleviating diarrhea, which is a key finding of this study.

In conclusion, our study provided evidence of the effectiveness and safety of P9 administration in alleviating chronic diarrhea. The diarrhea relief effect of P9 could be resulted from the modulation of specific intestinal functional bacteria (*Paraprevotella xylaniphila*, *Ruminococcus*_A *faecicola*, *Butyricicoccus*_A sp, *Acutalibacteraceae* sp, and *Streptococcus thermophilus*), bacteriophages (*Microviridae*, *Herelleviridae*, and *crAss-phage*), and intestinal metabolites (such as caffeic acid, taurine, cholate, chenodeoxycholate, acetic acid, butyric acid), thereby reducing intestinal inflammation and enhancing intestinal barrier function. The results of this study support the use of probiotics in managing chronic diarrhea.

MATERIALS AND METHODS

Subjects and trial design

This was a single-center, randomized, double-blind, placebo-controlled study, conducted in collaboration with the First Affiliated Hospital of Nanchang University from November 2020 to May 2021. A total of 189 patients with chronic diarrhea were enrolled in this study. The inclusion criteria were[67]: 1) age 18 to 65 years old; 2) loose or watery stools > 25% of bowel movements in the last three months, Bristol stool scale of types 5, 6, and 7; 3) symptom onset at least six months prior to enrollment; 4) routine fecal examination without white or red blood cell counts. The exclusion criteria included: 1) history of colon cancer, celiac disease, intestinal organic disease, or IBD; 2) pregnancy or lactation; 3) allergic to any ingredients in the intervention material; 4) treatment with antibiotics or probiotics within the last two weeks, or antidepressants, antianxiety agents, and other psychotropic drugs one month prior to enrolment; 5) long-term medication to improve diarrhea; 6) concurrent severe illness (such as myocardial infarction, cerebral infarction, malignant tumor and so on).

After a 2-week run-in period, patients were randomly divided into two groups: P9 (probiotic) and placebo. Blocked randomization of P9 and placebo treatment into a 1:1 ratio was achieved using a computer random sequence generator by an operator not involved in this study. The participants, doctors, and investigators involved in this study were blinded until after the primary data analysis. Finally, a total of 170 subjects completed the course of this study (n = 85 subjects per group; Fig. 1a).

The probiotic (P9) and placebo materials were manufactured by Jinhua Yinhe Biological Technology Co., Ltd. (Zhejiang, China). The P9 powder was prepared as dry powder of 1.0×10^{11} colony-forming units in 2 g. It was taken once a day 30 min after meal, mixed with warm water. The placebo powder was composed of maltodextrin. The probiotic and placebo materials were in identical shape, texture, and appearance. Subjects in each group received a 28-day supply of P9 or placebo material. The trial included a 14-day follow-up period after the intervention. Fecal samples were collected by sterile stool samplers and a DNA protection solution was added after sampling (Guangdong Longsee Biomedical Co., Ltd, Guangzhou, China) at day 0, 28, and 42. Samples were stored at -80°C before further analysis.

Endpoint definitions

The primary end point was the diarrhea symptom severity score assessed using the Gastrointestinal Symptom Rating Scale[68]; a higher score represents more severe symptoms. The scale consists of three main disease features: bowel frequency (score 0 to 3); urgency of defecation (score 0 to 3); stool consistency (score 0 to 3). The secondary end points included stool consistency (evaluated using the Bristol Stool Form scale, score 1 to 7), number of bowel movements, fecal urgency score, the Depression Anxiety Stress Scales-21 (DASS-21) [69], and fecal metagenome and metabolome[19]. The fecal urgency score was calculated from a four-point scale based on symptoms (0 = none, 1 = mild, 2 = moderate, and 3 = severe)[70]. Data were collected before and during the intervention by an electronic stool diary. The safety outcomes, including adverse events, were recorded throughout the course of intervention.

Fecal DNA extraction, shotgun metagenomic sequencing, and data quality control

Stool samples collected at days 0, 28 and 42 were shotgun sequenced (Illumina Novaseq 6000; Illumina Inc., San Diego, CA, USA). Metagenomic DNA was extracted using the Magnetic Soil and Stool DNA Kit (DP712; TIANGEN Biotech Co., Ltd., Beijing, China) from fecal samples following previously reported procedures[25, 71].

A total of 11.49 Tbp (mean = 23.21 Gbp/sample; Table S1) high-quality clean data were generated. Then, the clean data of samples were assembled into contigs using MEGAHIT[72], and contigs larger than 2,000 bp were screened for binning using MetaBAT2[73], VAMB[74], and DAS Tool[75] with default parameters. Finally, all bins were combined to generate metagenome-assembled genomes (MAGs) using in-house scripts.

As previously reported[71], the quality of MAGs were examined using CheckM[76] and classified into high-, medium-, and partial-quality based on their completeness and contamination. The high-quality genomes were clustered, and then the most representative genomes from each replicate set were selected to obtain species-level genome bins (SGBs) by dRep (parameters: -pa 0.95 and -sa 0.95)[77]. Taxonomic annotation of SGBs were performed by aligning genomes to a curated set of 6,530 representative bacterial genomes from the National Center for Biotechnology Information Non-Redundant Protein Sequence Database using Kraken2. The relative abundance of each SGB was calculated by CoverM (https://github.com/wwood/CoverM, parameters: –min-read-percent-identity 0.95 –min-covered-fraction 0.4).

Published literature and MetaCyc metabolic database were used to predict SGBs encoding related gut metabolic modules (GMMs)[78, 79]. The open reading frames were compared to the Kyoto Encyclopedia of Genes and Genomes Orthologies database to annotate the key metabolic modules of each SGB, and the distribution of synthesis or degradation modules in the SGBs were identified by Omixer-RPM (parameter: -c 0.66). In addition, the distribution of gut bioactive metabolic compounds was predicted based on our previous work[80]. Carbohydrate-active enzymes (CAZymes)-encoding genes were further detected using dbCAN2[81].

Phageome identification and abundance analysis

All assembled contigs were firstly assessed by CheckV, then potential viral sequences were identified by VIBRANT with default options[82, 83]. Proposed viral contigs greater than 5,000 bp were recognized and further clustered into viral operational taxonomic units (vOTUs) with 95% nucleotide identity across 80% of the sequence using CD-HIT (https://github.com/weizhongli/cdhit)[84]. Then, 145,589 vOTUs were cross-compared to a curated set of 189,680 viral genomes from the Metagenomic Gut Virus catalogue (accessed July 2021) for evaluating the novelty of vOTUs in this dataset[85]. The average abundance of

vOTUs across the viral population was finally calculated using the CoverM-contig pipeline (https://github.com/wwood/CoverM, parameters: –min-read-percent-identity 0.95, –min-read-aligned-percent 0.5, –proper-pairs-only, and – exclude-supplementary).

Fecal metabolomics

Fecal samples were dried and then mixed with 600 μL of methanol solution containing 2-chlorophenylalanine. The mixture was vortexed for 30 s, before grinding for 60 s at 55 Hz in the presence of 100 mg glass beads. After ultrasonification and centrifuging at 12,000 rpm for 10 min, the supernatant was filtered through a 0.22 μm ultrafiltration membrane. Chromatographic separations were performed according to a previous work[25]. Briefly, separation was carried out on an ACQUITY UPLC BEH amide column (100 × 2.1 mm, 1.7 μm; Waters Corporation, Milford, MA, USA) at 25°C and at a flow rate of 0.5 mL/ min. A mixture of 25 mM ammonium acetate and 25 mM ammonia in water (A) and acetonitrile (B) were used as mobile phases. Each sample was analyzed in both positive and negative ionization modes under electrospray ionization.

The ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry raw data were first processed by ProteoWizard software (version 3.0.5047) and the R package XCMS. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were applied to identify differential biomarker metabolites between groups (cut-off level: variable importance in projection (VIP) value > 2 and *P* value < 0.05). Further identification of selected biomarkers and potential metabolic pathways was performed by searching through HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/), Massbank (http://www.massbank.jp), and KEGG (http://www.kegg.com/) databases.

The fecal SCFA levels of 26 samples (from 13 randomly chosen subjects per group) were determined by gas chromatography mass spectrometry (GC-MS). Firstly, stool samples (50 mg) were weighed and transferred to clean centrifuge tubes, and 15% phosphoric acid (50 μL), 75 μg/mL internal standard (isocaproic acid, 10 μL), and diethyl ether (140 μL) were added. Then, the solutions were mixed for 1 min and centrifuged at 12,000 rpm for 10 min at 4 ℃. Afterwards, an aliquot of 200 μL of the supernatant was transferred to a sample vial for analysis. The GC-MS conditions were set according to the conditions described in a previous study[86].

Statistical analyses

Statistical tests and data visualization were performed with R software (v.4.1.0) and Adobe Illustrator; and all data are expressed in mean ± SEM. The R packages, including vegan, optparse, mixOmics, ggplot2, and ggpubr, were used to calculate the Shannon and Simpson's diversity index and execute PCA, principal coordinate analysis (PCoA), PLS-DA, adonis test, and Procrustes analysis. Wilcoxon test and t-test were used to evaluate statistical difference in various variables between groups, and the Benjamini-

Hochberg procedure was used to adjust the *P* value for multiple comparison. $P < 0.05$ was considered to be significant. Pearson correlation analysis was used to analyze the association between clinical indicators, fecal bacteria, and metabolites.

Declarations

Data sharing statement

Sequencing data are available in the China National GeneBank (https://db.cngb.org/cnsa/; accession number: CNP0004247).

Declaration of interests

All authors declare no competing interests.

References

1. Burgers K, Lindberg B, Bevis ZJ. Chronic Diarrhea in Adults: Evaluation and Differential Diagnosis. *American family physician* **101**, 472-480 (2020).
2. Chu C, Rotondo-Trivette S, Michail S. Chronic diarrhea. *Current problems in pediatric and adolescent health care* **50**, 100841 (2020).
3. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* **130**, 1480-1491 (2006).
4. Schiller LR, Pardi DS, Sellin JH. Chronic Diarrhea: Diagnosis and Management. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* **15**, 182-193.e183 (2017).
5. Ma H, et al. The effect of clonidine on gastrointestinal side effects associated with ultra-rapid opioid detoxification. *Anesthesia and analgesia* **96**, 1409-1412 (2003).
6. Wang Y, et al. Probiotics therapy for adults with diarrhea-predominant irritable bowel syndrome: a systematic review and meta-analysis of 10 RCTs. *International journal of colorectal disease* **37**, 2263-2276 (2022).
7. Wang Y, et al. Antidepressants can induce mutation and enhance persistence toward multiple antibiotics. *Proceedings of the National Academy of Sciences of the United States of America* **120**, e2208344120 (2023).
8. Li Y, et al. Gut Microbiota and Diarrhea: An Updated Review. *Frontiers in cellular and infection microbiology* **11**, 625210 (2021).
9. Rouhani S, et al. Diarrhea as a Potential Cause and Consequence of Reduced Gut Microbial Diversity Among Undernourished Children in Peru. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **71**, 989-999 (2020).

10. Chen P, et al. Modulation of gut mucosal microbiota as a mechanism of probiotics-based adjunctive therapy for ulcerative colitis. *Microbial biotechnology* **13**, 2032-2043 (2020).
11. Zhang WX, et al. Altered profiles of fecal metabolites correlate with visceral hypersensitivity and may contribute to symptom severity of diarrhea-predominant irritable bowel syndrome. *World journal of gastroenterology* **25**, 6416-6429 (2019).
12. Cao Z, Sugimura N, Burgermeister E, Ebert MP, Zuo T, Lan P. The gut virome: A new microbiome component in health and disease. *EBioMedicine* **81**, 104113 (2022).
13. Duan Y, Young R, Schnabl B. Bacteriophages and their potential for treatment of gastrointestinal diseases. *Nature reviews Gastroenterology & hepatology* **19**, 135-144 (2022).
14. Hill C, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature reviews Gastroenterology & hepatology* **11**, 506-514 (2014).
15. Bron PA, et al. Can probiotics modulate human disease by impacting intestinal barrier function? *The British journal of nutrition* **117**, 93-107 (2017).
16. Gu Y, et al. Saccharomyces boulardii, a yeast probiotic, inhibits gut motility through upregulating intestinal serotonin transporter and modulating gut microbiota. *Pharmacological research* **181**, 106291 (2022).
17. Wu Y, et al. Effect of a Multispecies Probiotic Mixture on the Growth and Incidence of Diarrhea, Immune Function, and Fecal Microbiota of Pre-weaning Dairy Calves. *Frontiers in microbiology* **12**, 681014 (2021).
18. Yang B, et al. Lactobacillus plantarum CCFM1143 Alleviates Chronic Diarrhea via Inflammation Regulation and Gut Microbiota Modulation: A Double-Blind, Randomized, Placebo-Controlled Study. *Frontiers in immunology* **12**, 746585 (2021).
19. Skrzydło-Radomańska B, et al. The Effectiveness and Safety of Multi-Strain Probiotic Preparation in Patients with Diarrhea-Predominant Irritable Bowel Syndrome: A Randomized Controlled Study. *Nutrients* **13**, (2021).
20. Jung M, Jung S, Kim N, Ahn H, Yun H, Kim KN. A Randomized, Double-Blind, Placebo-Controlled Trial to Assess the Efficacy and Safety of Lactiplantibacillus plantarum CJLP243 in Patients with Functional Diarrhea and High Fecal Calprotectin Levels. *Nutrients* **14**, (2022).
21. Simrén M, et al. Clinical trial: the effects of a fermented milk containing three probiotic bacteria in patients with irritable bowel syndrome - a randomized, double-blind, controlled study. *Alimentary pharmacology & therapeutics* **31**, 218-227 (2010).
22. Li C, et al. Screening for Lactobacillus plantarum Strains That Possess Organophosphorus Pesticide-Degrading Activity and Metabolomic Analysis of Phorate Degradation. *Frontiers in microbiology* **9**, 2048 (2018).
23. Hai, et al. Screening for Lactobacillus plantarum with potential inhibitory activity against enteric pathogens. (2014).

24. Liu W, et al. Lactiplantibacillus plantarum P9 improved gut microbial metabolites and alleviated inflammatory response in pesticide exposure cohorts. *iScience* **25**, 104472 (2022).
25. Ma T, et al. Effect of the probiotic strain, Lactiplantibacillus plantarum P9, on chronic constipation: A randomized, double-blind, placebo-controlled study. *Pharmacological research* **191**, 106755 (2023).
26. Schiller LR. Evaluation of chronic diarrhea and irritable bowel syndrome with diarrhea in adults in the era of precision medicine. *The American journal of gastroenterology* **113**, 660-669 (2018).
27. Maity C, Gupta AK. Therapeutic efficacy of probiotic Alkalihalobacillus clausii 088AE in antibiotic-associated diarrhea: A randomized controlled trial. *Heliyon* **7**, e07993 (2021).
28. McFarland LV, Goh S. Are probiotics and prebiotics effective in the prevention of travellers' diarrhea: A systematic review and meta-analysis. *Travel medicine and infectious disease* **27**, 11-19 (2019).
29. Szajewska H, Kołodziej M, Zalewski BM. Systematic review with meta-analysis: Saccharomyces boulardii for treating acute gastroenteritis in children-a 2020 update. *Alimentary pharmacology & therapeutics* **51**, 678-688 (2020).
30. Bai T, et al. The Short-term Efficacy of Bifidobacterium Quadruple Viable Tablet in Patients with Diarrhea-Predominant Irritable Bowel Syndrome: Potentially Mediated by Metabolism rather than Diversity Regulation. *The American journal of gastroenterology*, (2022).
31. Xu H, et al. Metagenomic analysis revealed beneficial effects of probiotics in improving the composition and function of the gut microbiota in dogs with diarrhoea. *Food & function* **10**, 2618-2629 (2019).
32. Lin L, et al. Genome-centric investigation of bile acid metabolizing microbiota of dairy cows and associated diet-induced functional implications. *The ISME journal* **17**, 172-184 (2023).
33. Morotomi M, Nagai F, Sakon H, Tanaka R. Paraprevotella clara gen. nov., sp. nov. and Paraprevotella xylaniphila sp. nov., members of the family 'Prevotellaceae' isolated from human faeces. *International journal of systematic and evolutionary microbiology* **59**, 1895-1900 (2009).
34. Sun H, et al. Probiotics synergized with conventional regimen in managing Parkinson's disease. *npj Parkinson's Disease* **8**, 62 (2022).
35. Liu Z, et al. Bacillus coagulans in Combination with Chitooligosaccharides Regulates Gut Microbiota and Ameliorates the DSS-Induced Colitis in Mice. *Microbiology spectrum* **10**, e0064122 (2022).
36. Geirnaert A, et al. Butyrate-producing bacteria supplemented in vitro to Crohn's disease patient microbiota increased butyrate production and enhanced intestinal epithelial barrier integrity. *Scientific reports* **7**, 11450 (2017).
37. Lyra A, et al. Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification. *World journal of gastroenterology* **15**, 5936-5945 (2009).
38. Crost EH, Coletto E, Bell A, Juge N. Ruminococcus gnavus: friend or foe for human health. *FEMS Microbiology Reviews* **47**, (2023).
39. Li S, Qi Y, Ren D, Qu D, Sun Y. The Structure Features and Improving Effects of Polysaccharide from Astragalus membranaceus on Antibiotic-Associated Diarrhea. *Antibiotics (Basel, Switzerland)* **9**,

(2019).

40. Auer IO, Röder A, Wensinck F, van de Merwe JP, Schmidt H. Selected bacterial antibodies in Crohn's disease and ulcerative colitis. *Scandinavian journal of gastroenterology* **18**, 217-223 (1983).
41. Yang H, et al. Quyushengxin Formula Causes Differences in Bacterial and Phage Composition in Ulcerative Colitis Patients. *Evidence-based complementary and alternative medicine : eCAM* **2020**, 5859023 (2020).
42. Mihindukulasuriya KA, et al. Multi-Omics Analyses Show Disease, Diet, and Transcriptome Interactions With the Virome. *Gastroenterology* **161**, 1194-1207.e1198 (2021).
43. Zuo W, Michail S, Sun F. Metagenomic Analyses of Multiple Gut Datasets Revealed the Association of Phage Signatures in Colorectal Cancer. *Frontiers in cellular and infection microbiology* **12**, 918010 (2022).
44. Gold A, Zhu J. Not just a gut feeling: a deep exploration of functional bacterial metabolites that can modulate host health. *Gut microbes* **14**, 2125734 (2022).
45. Farrugia A, Arasaradnam R. Bile acid diarrhoea: pathophysiology, diagnosis and management. *Frontline gastroenterology* **12**, 500-507 (2021).
46. Sinha SR, et al. Dysbiosis-Induced Secondary Bile Acid Deficiency Promotes Intestinal Inflammation. *Cell host & microbe* **27**, 659-670.e655 (2020).
47. Lowes S, Simmons NL. Human intestinal cell monolayers are preferentially sensitive to disruption of barrier function from basolateral exposure to cholic acid: correlation with membrane transport and transepithelial secretion. *Pflugers Archiv : European journal of physiology* **443**, 265-273 (2001).
48. Odunsi-Shiyanbade ST, et al. Effects of chenodeoxycholate and a bile acid sequestrant, colesevelam, on intestinal transit and bowel function. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* **8**, 159-165 (2010).
49. Brass EP, Stelten BML, Verrips A. Cerebrotendinous xanthomatosis-associated diarrhea and response to chenodeoxycholic acid treatment. *JIMD reports* **56**, 105-111 (2020).
50. Mishima Y, Ishihara S. Molecular Mechanisms of Microbiota-Mediated Pathology in Irritable Bowel Syndrome. *International journal of molecular sciences* **21**, (2020).
51. Li P, et al. Characterization of kynurenine pathway in patients with diarrhea-predominant irritable bowel syndrome. *European journal of histochemistry : EJH* **64**, (2020).
52. Ahmadi S, et al. A human-origin probiotic cocktail ameliorates aging-related leaky gut and inflammation via modulating the microbiota/taurine/tight junction axis. *JCI insight* **5**, (2020).
53. Shimizu M, Zhao Z, Ishimoto Y, Satsu H. Dietary taurine attenuates dextran sulfate sodium (DSS)-induced experimental colitis in mice. *Advances in experimental medicine and biology* **643**, 265-271 (2009).
54. Fortin G. L-Carnitine and intestinal inflammation. *Vitamins and hormones* **86**, 353-366 (2011).
55. Kim SY, Seo YS, Lee ES, Kim KN. Total Bilirubin Is Associated with Small Intestinal Bacterial Overgrowth in Diarrhea Predominant Irritable Bowel Syndrome. *Annals of clinical and laboratory*

science **49**, 344-352 (2019).

56. Wan F, et al. Caffeic Acid Supplement Alleviates Colonic Inflammation and Oxidative Stress Potentially Through Improved Gut Microbiota Community in Mice. *Frontiers in microbiology* **12**, 784211 (2021).
57. Calzada F, Correa-Basurto J, Barbosa E, Mendez-Luna D, Yepez-Mulia L. Antiprotozoal Constituents from Annona cherimola Miller, a Plant Used in Mexican Traditional Medicine for the Treatment of Diarrhea and Dysentery. *Pharmacognosy magazine* **13**, 148-152 (2017).
58. Pujo J, et al. Bacteria-derived long chain fatty acid exhibits anti-inflammatory properties in colitis. *Gut* **70**, 1088-1097 (2021).
59. Wardman JF, Bains RK, Rahfeld P, Withers SG. Carbohydrate-active enzymes (CAZymes) in the gut microbiome. *Nature reviews Microbiology* **20**, 542-556 (2022).
60. El Kaoutari A, et al. Development and validation of a microarray for the investigation of the CAZymes encoded by the human gut microbiome. *PloS one* **8**, e84033 (2013).
61. Lan Q, et al. Association of gut microbiota and SCFAs with finishing weight of Diannan small ear pigs. *Frontiers in microbiology* **14**, 1117965 (2023).
62. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. *Advances in immunology* **121**, 91-119 (2014).
63. Martin-Gallausiaux C, Marinelli L, Blottière HM, Larraufie P, Lapaque N. SCFA: mechanisms and functional importance in the gut. *The Proceedings of the Nutrition Society* **80**, 37-49 (2021).
64. Laffin M, et al. A high-sugar diet rapidly enhances susceptibility to colitis via depletion of luminal short-chain fatty acids in mice. *Scientific reports* **9**, 12294 (2019).
65. Gąsiorowska A, et al. Effects of Microencapsulated Sodium Butyrate, Probiotics and Short Chain Fructooligosaccharides in Patients with Irritable Bowel Syndrome: A Study Protocol of a Randomized Double-Blind Placebo-Controlled Trial. *Journal of clinical medicine* **11**, (2022).
66. Plöger S, et al. Microbial butyrate and its role for barrier function in the gastrointestinal tract. *Annals of the New York Academy of Sciences* **1258**, 52-59 (2012).
67. Mearin F, et al. Bowel Disorders. *Gastroenterology*, (2016).
68. Svedlund J, Sjödin I, Dotevall G. GSRS--a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. *Digestive diseases and sciences* **33**, 129-134 (1988).
69. Lovibond PF, Lovibond SH. The structure of negative emotional states: comparison of the Depression Anxiety Stress Scales (DASS) with the Beck Depression and Anxiety Inventories. *Behaviour research and therapy* **33**, 335-343 (1995).
70. Plasse TF, et al. Bimodal Release Ondansetron Improves Stool Consistency and Symptomatology in Diarrhea-Predominant Irritable Bowel Syndrome: A Randomized, Double-Blind, Trial. *The American journal of gastroenterology* **115**, 1466-1473 (2020).

71. Sun B, et al. Bifidobacterium lactis Probio-M8 Adjuvant Treatment Confers Added Benefits to Patients with Coronary Artery Disease via Target Modulation of the Gut-Heart/-Brain Axes. *mSystems* **7**, e0010022 (2022).
72. Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics (Oxford, England)* **31**, 1674-1676 (2015).
73. Kang DD, et al. MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* **7**, e7359 (2019).
74. Nissen JN, et al. Improved metagenome binning and assembly using deep variational autoencoders. *Nature biotechnology* **39**, 555-560 (2021).
75. Sieber CMK, et al. Recovery of genomes from metagenomes via a dereplication, aggregation and scoring strategy. *Nature microbiology* **3**, 836-843 (2018).
76. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome research* **25**, 1043-1055 (2015).
77. Olm MR, Brown CT, Brooks B, Banfield JF. dRep: a tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication. *The ISME journal* **11**, 2864-2868 (2017).
78. Skonieczna-Żydecka K, et al. Gut Biofactory-Neurocompetent Metabolites within the Gastrointestinal Tract. A Scoping Review. *Nutrients* **12**, (2020).
79. James SC, Fraser K, Young W, McNabb WC, Roy NC. Gut Microbial Metabolites and Biochemical Pathways Involved in Irritable Bowel Syndrome: Effects of Diet and Nutrition on the Microbiome. *The Journal of nutrition* **150**, 1012-1021 (2020).
80. Liu A, et al. Adjunctive Probiotics Alleviates Asthmatic Symptoms via Modulating the Gut Microbiome and Serum Metabolome. *Microbiology spectrum* **9**, e0085921 (2021).
81. Zhang H, et al. dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic acids research* **46**, W95-w101 (2018).
82. Kieft K, Zhou Z, Anantharaman K. VIBRANT: automated recovery, annotation and curation of microbial viruses, and evaluation of viral community function from genomic sequences. *Microbiome* **8**, 90 (2020).
83. Nayfach S, Camargo AP, Schulz F, Eloe-Fadrosh E, Roux S, Kyrpides NC. CheckV assesses the quality and completeness of metagenome-assembled viral genomes. *Nature biotechnology* **39**, 578-585 (2021).
84. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics (Oxford, England)* **28**, 3150-3152 (2012).
85. Nayfach S, et al. Metagenomic compendium of 189,680 DNA viruses from the human gut microbiome. *Nature microbiology* **6**, 960-970 (2021).

86. Su SH, et al. Fecal microbiota transplantation and short-chain fatty acids protected against cognitive dysfunction in a rat model of chronic cerebral hypoperfusion. *CNS neuroscience & therapeutics*, (2023).

Figures

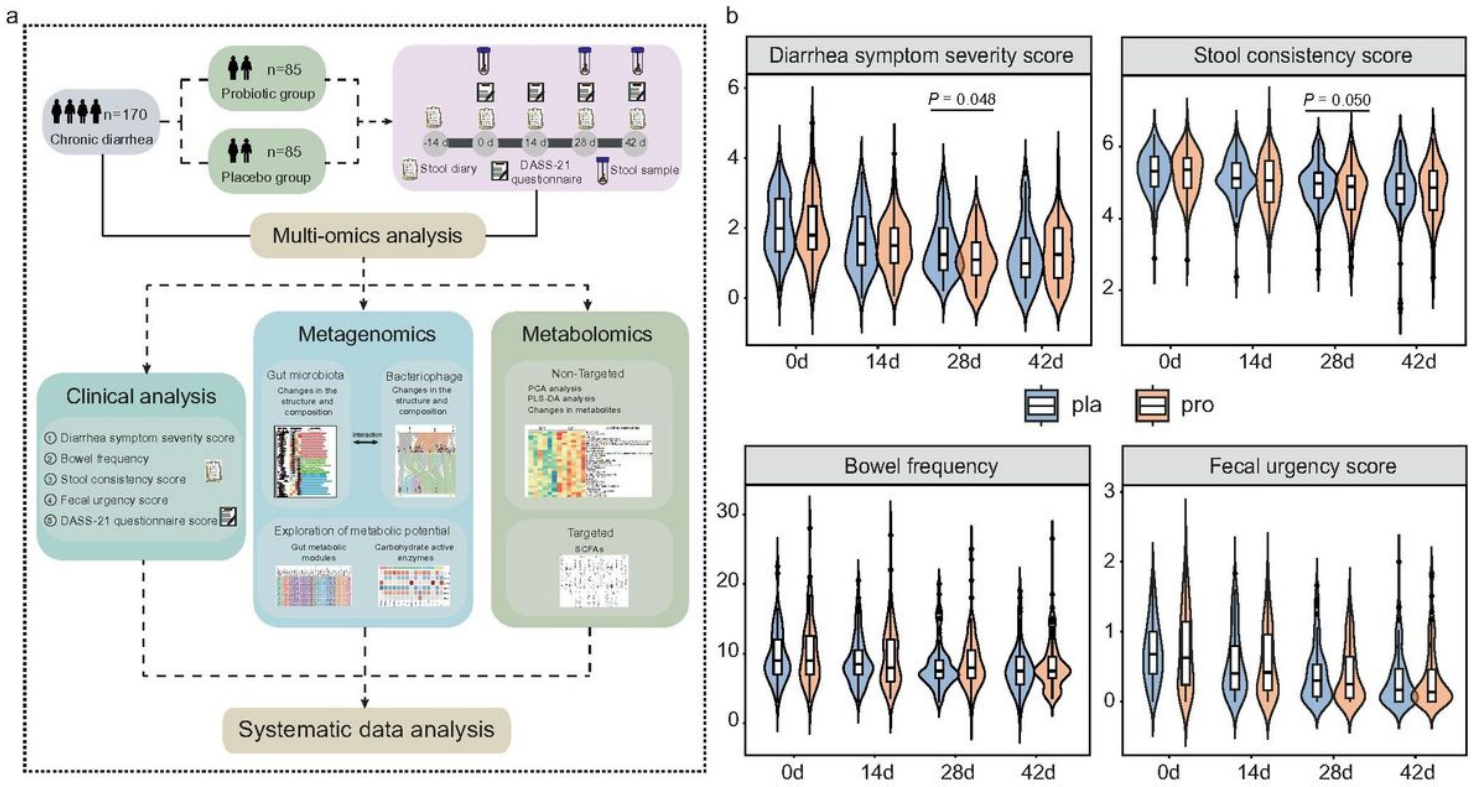

Figure 1

Multi-omics analysis pipeline and clinical indicators of diarrhea symptoms. (a) The workflow of trial design. Analysis of clinical indicators, fecal metagenomics and metabolomics. (b) Changes in the diarrhea symptom severity score, stool consistency score, bowel frequency, and fecal urgency score in the P9 (pro) and placebo (pla) groups, evaluated by Wilcoxon test. Significant differences are shown in the plots. “0d”, “14d”, “28d”, and “42d” represent the baseline before the intervention, 14, 28 and 42 days after intervention, respectively. DASS-21 = Depression Anxiety Stress Scales-21; PCA = principal component analysis; PLS-DA = partial least squares-discriminant analysis; SCFAs = short-chain fatty acids.

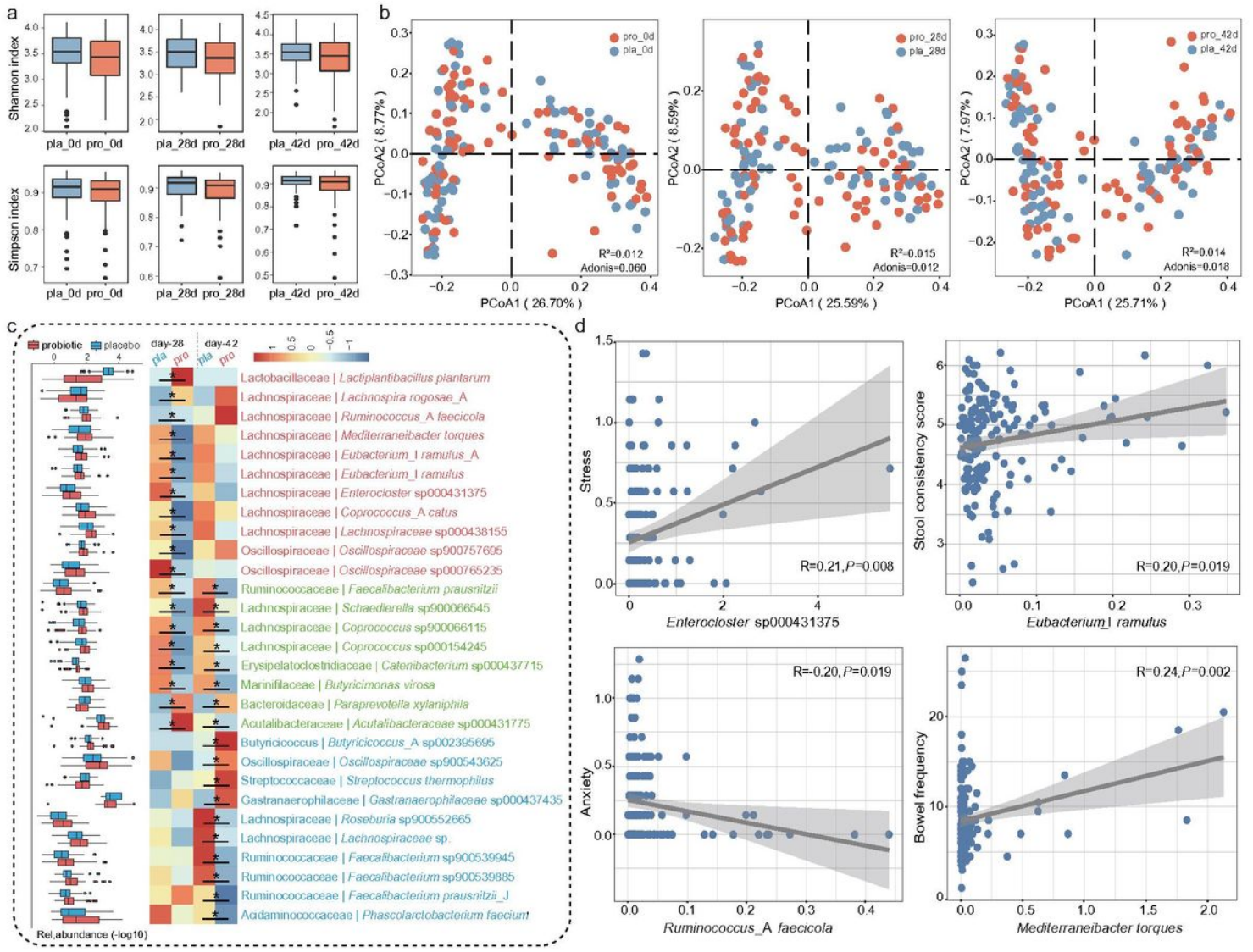

Figure 2

Microbial diversity and differentially abundant species-level genome bins (SGBs) between groups and their correlation with clinical indicators of diarrhea. (a) Shannon and Simpson diversity indices and (b) principal coordinates analysis (PCoA) score plots of the gut microbiota of the placebo (pla) and P9 (pro) groups at days 0 (0d), 28 (28d), and 42 (42d). Symbols representing samples of the two groups at different time points are shown in different colors. Results of Adonis tests are shown at the right lower corner of the PCoA plots. (c) Significant differential SGBs between the P9 and placebo groups at different time points. The red, green and blue fonts represent bacteria that changed significantly between the two groups at days 28, days 28 and 42, and days 42, respectively ($P < 0.05$, Wilcoxon test). (d) Pearson correlation scatter plots showed the correlation between differentially abundant SGBs and clinical indicators of diarrhea of the P9 group after 28-day intervention. The grey straight line represents the fitted values, and the shaded area indicates 95% confidence interval.

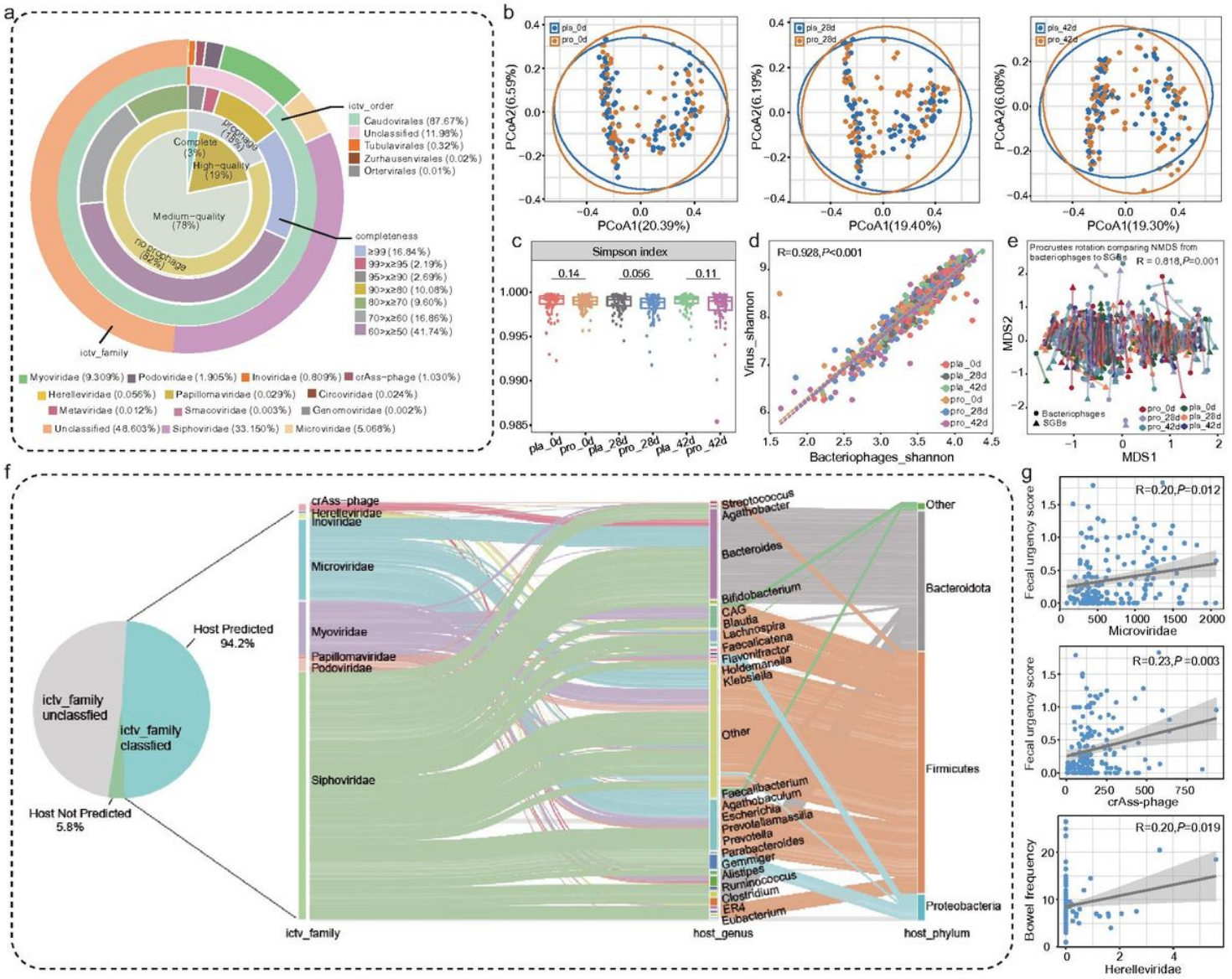

Figure 3

The gut phageome of patients in P9 (pro) and placebo (pla) groups during/after the intervention. (a) The circle diagram shows the quality level and completeness of annotated virus operational taxonomic units (vOTUs; the second layer of circle from the inside to the outside), whether they were classified as “prophage” or “non-prophage” (the first layer of circl), and their order- and family-level taxonomic distribution according to the International Committee on Taxonomy of Viruses classification (ICTV classification; the third and fourth layers of circle, respectively). (b) Principal coordinates analysis (PCoA) and (c) Simpson’s diversity index of the gut microbiota of the two groups at days 0 (0d), 28 (28d), and 42 (42d). (d) Pearson correlation between the Shannon diversity index of the gut bacterial microbiota and phageome; and a strong positive correlation was found (R = 0.928; $P < 0.001$). (e) Procrustes analysis performed on the gut species-level genome bins (SGB) and bacteriophages of the two groups at different time points confirmed a positive cooperativity between the gut bacterial microbiota and phageome (correlation = 0.818; $P = 0.001$). (f) Family-level classification of phages and their bacterial hosts at the genus and phylum levels. (g) Pearson correlation scatter plots showed the correlation between family-

level bacteriophages and clinical indicators of diarrhea of the P9 group after 28-day intervention. The grey straight line represents the fitted values, and the shaded area indicates 95% confidence interval.

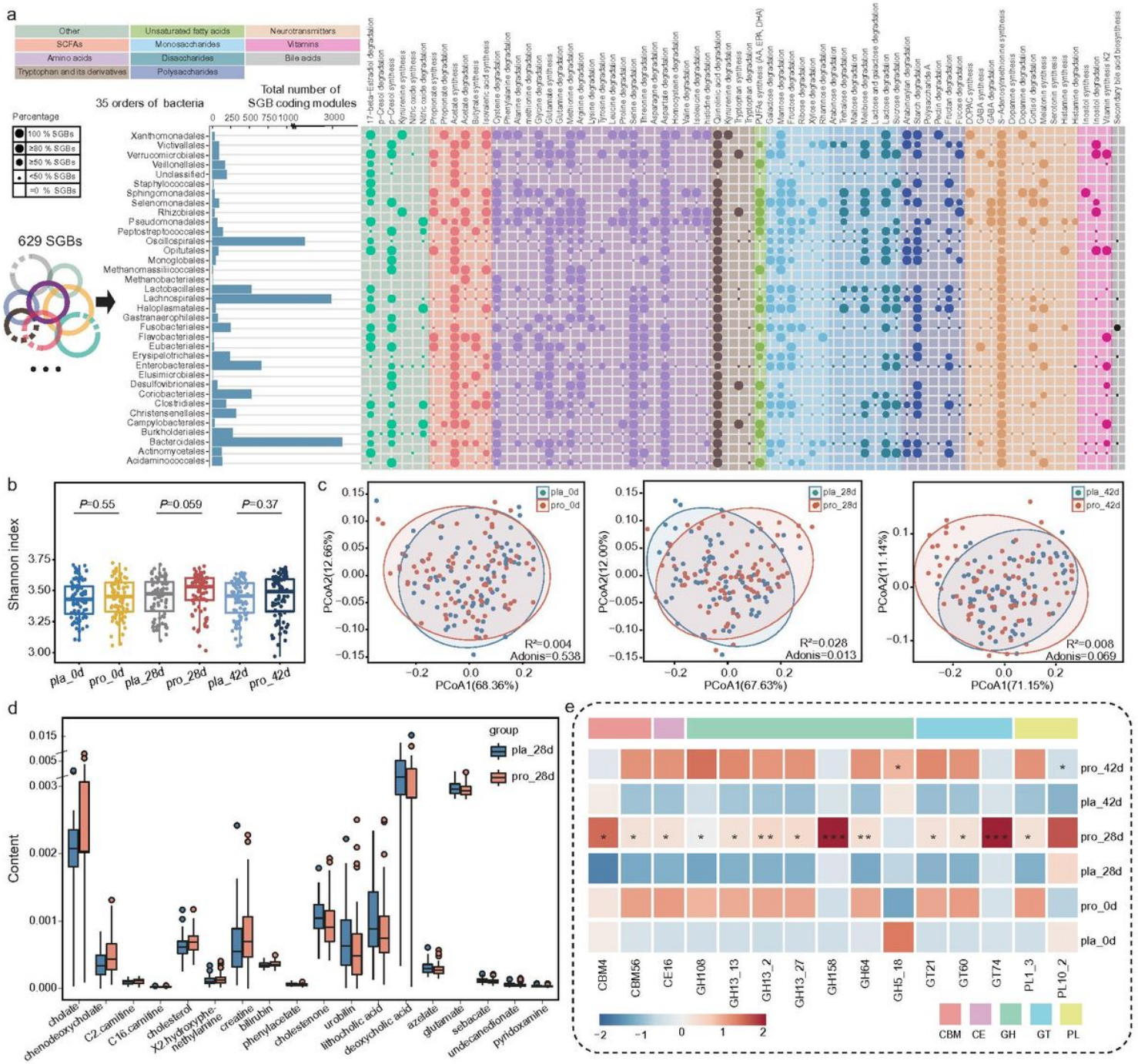

Figure 4

Changes in the predicted gut metabolic modules (GMMs), gut bioactive metabolites, and carbohydrate active enzymes (CAZymes) in P9 (pro) and placebo (pla) groups during the trial. (a) Distribution of 72 identified GMMs (belonging to 11 metabolic modules, including SCFAs, amino acids, tryptophan and its derivatives, unsaturated fatty acids, monosaccharides, disaccharides, polysaccharides, neurotransmitters, vitamins, bile acids, and other metabolic modules) across 34 bacterial orders (corresponding to 629 types

of species-level genome bins, SGBs). Each metabolic module is shown in a different color. The size of the circle in each module represents the proportion of SGBs encoding that specific module. (b) Shannon diversity index and (c) principal coordinates analysis (PCoA) score plots of the gut microbiota of the two groups at days 0 (0d), 28 (28d), and 42 (42d). Results of Adonis tests are shown at the right lower corner of PCoA plots. (d) Boxplots comparing the abundances of predicted differential bioactive metabolites that were responsive to the P9 treatment at day 28 of intervention. (e) Heatmap showing the enrichment of specific families of CAZyme (CBM, CE, GH, GT, and PL) in the fecal metagenome of the two groups at days 28 and 42. CAZyme = carbohydrate-active enzyme, CBM = carbohydrate-binding modules, CE = carbohydrate esterases, GH = glycoside hydrolases, GT = glycosyltransferases, PL = polysaccharide lyases. The color scale represents relative distribution, ranging from -2 (low abundance) to 2 (high abundance). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, evaluated by Wilcoxon test.

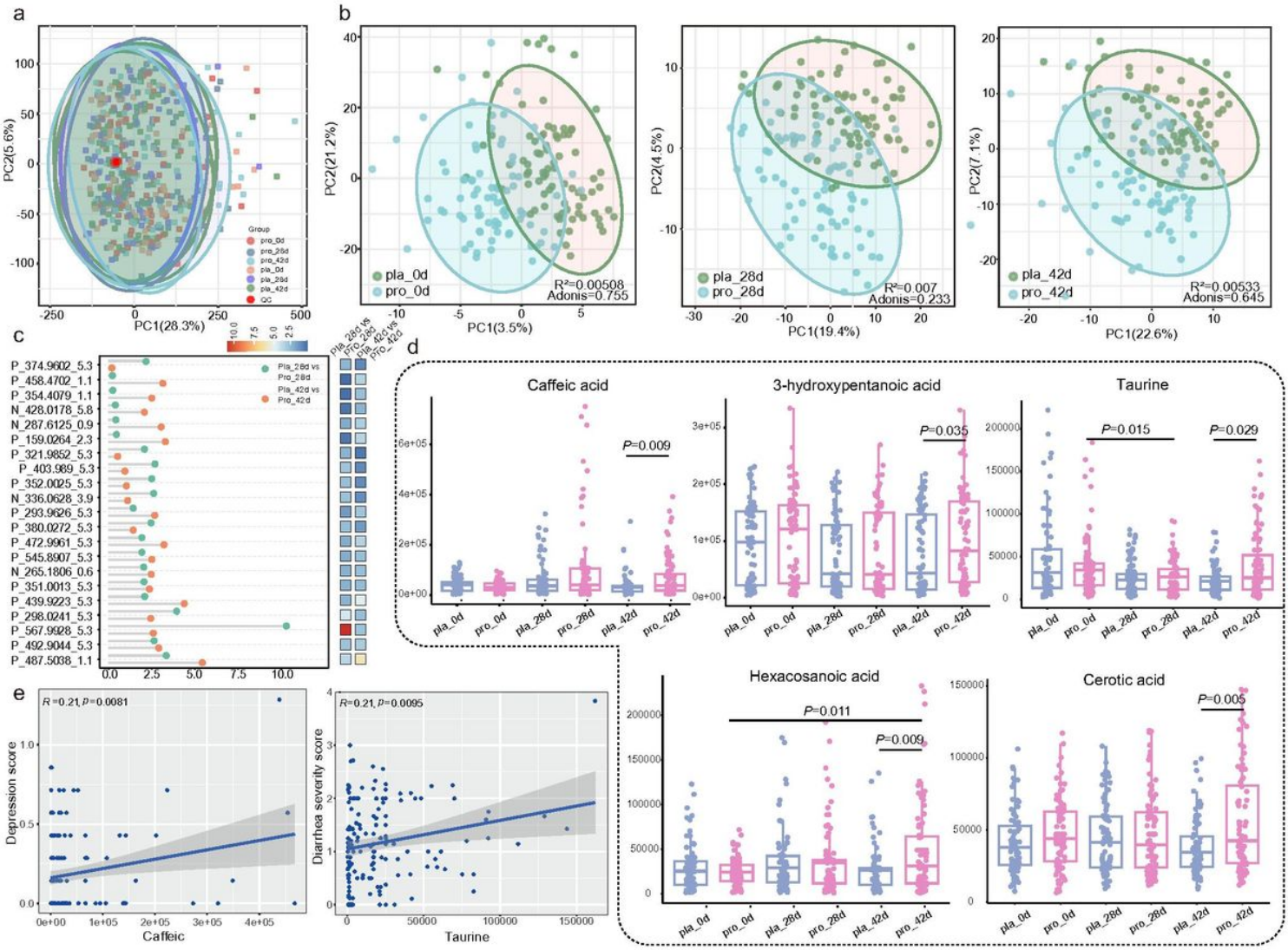

Figure 5

Changes in the fecal metabolome of patients in P9 (pro) and placebo (pla) groups during/after the intervention. (a) Principal component analysis (PCA) and (b) partial least squares-discriminant analysis (PLS-DA) score scatter plots of the fecal metabolome of two groups at days 0 (0d), 28 (28d), and 42

(42d) of intervention. Results of Adonis test are shown at the right lower corner of PCoA plots. QC represents quality control samples. (c) Significant differential fecal metabolites identified between the two groups at days 28 and 42. The variable importance in projection (VIP) scores were generated by PLS-DA to identify the metabolite contribution to the discrimination (cut-off: VIP > 2; $P < 0.05$). (d) Boxplots showing relevant P9-intervention responsive fecal metabolites. Significant differences between groups are shown (evaluated by Wilcoxon test, corrected by Benjamini-Hochberg procedure; corrected $P < 0.05$ was considered statistically significant). (e) Pearson correlation scatter plots showed the correlation between differential fecal metabolites and clinical indicators of diarrhea of the P9 group after 28-day intervention. The grey straight line represents the fitted values, and the shaded area indicates 95% confidence interval.

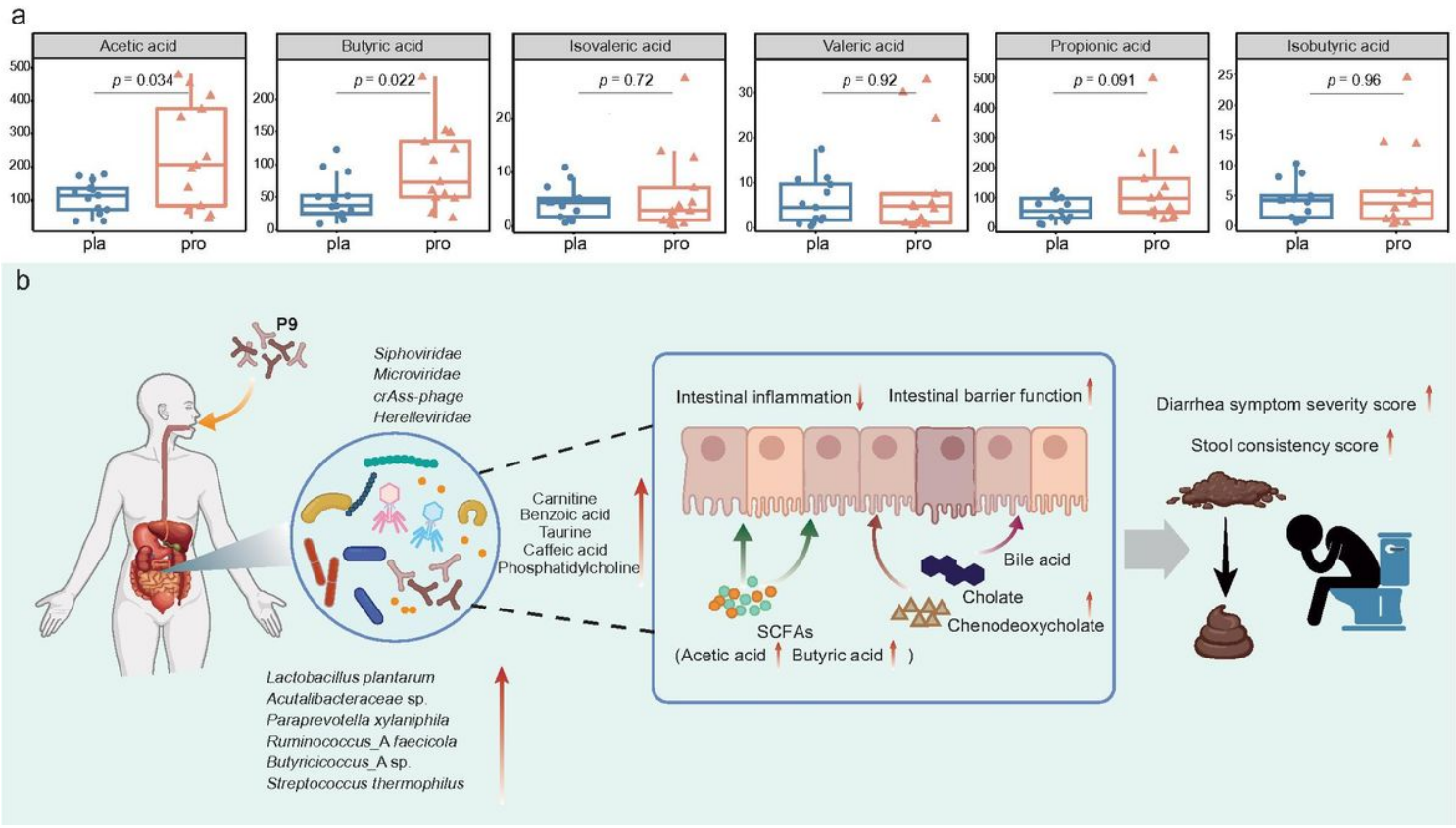

Figure 6

Fecal short-chain fatty acids (SCFAs) in P9 (pla) and placebo (pla) groups and schematic diagram of beneficial effects of administering *Lactiplantibacillus plantarum* P9 (P9) in chronic diarrhea. (a) Significant differences in the concentrations of fecal SCFAs between the two groups at days 28. $P < 0.05$ (Wilcoxon test) was considered statistically significant. (b) Schematic diagram showing P9-driven changes in host gut microbiota and metabolites, which might have contributed to diarrhea relief.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.pdf
- Supplementarytable.pdf