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Papaya polysaccharides regulate intestinal flora *in vivo*

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Abstract

Mainly, papaya is a fruit grown in tropical and subtropical regions. It is a nutritionally rich fruit that is widely recognized for its various benefits. There are some common benefits of papaya include improvement of digestion, abundant vitamin C, immune system boosts, promotion of cardiovascular health, support of eye health, enhance of gut health etc. Papaya polysaccharides (PP) are natural polysaccharide compounds extracted from papaya. They are complex compounds composed of polysaccharide molecules obtained from papaya pulp, peel, or juice. PP are believed to possess various pharmacological activities and health benefits include immunomodulation, antioxidant effects, anti-inflammatory effects, blood glucose-lowering effects, hepatoprotective effects etc. It is important to note that although papaya polysaccharides have potential health benefits, current research on them is still relatively limited. Further scientific studies are needed to validate and deepen our understanding of their mechanisms of action and their application in different disease conditions. Therefore, we focused on the effects of PP regulate intestinal flora *in vivo* in this study. The results were revealed that long-read sequencing platform, Oxford Nanopore Technologies (ONT), was used to classify the gut microbiota in rat fecal samples. A total of 945 bacterial strains were identified through comprehensive strain identification. The obtained sequencing reads were analyzed using the CLC Genomics Workbench software. Moreover, CLC Genomics Workbench software performed Weighted UniFrac and Bray-Curtis analyses to measure the dissimilarity of identified bacterial species between different groups. Subsequently, the PERMANOVA statistical method was employed to determine the significance of differences in the composition of bacterial species between groups. Comparing the relative abundance changes of identified bacterial species in the fecal samples after 28 days of low-dose PP consumption [100 mg/kg body weight (BW)] with the normal diet group, 46 intestinal bacterial strains showed statistically significant differences. After comparing the relative abundance changes of identified bacterial species in the fecal samples following 28 days of high-dose PP consumption (200 mg/kg BW) with the normal diet group, 45 intestinal bacterial strains showed statistically significant differences. Using CLC Genomics Workbench software, a heat map was generated by selecting bacterial strains in the high (28) group that exhibited a relative abundance increase or decrease of more than 4-fold. After database analysis, a total of 945 intestinal bacterial strains were identified in this study. It was observed that the proportions of intestinal bacterial communities changed after consuming PP. Among them, 42 bacterial strains showed an increase in abundance after PP consumption. According to the criteria for determining good and bad bacteria, out of the total 118 bacterial genera considered, 69 belong to the "good bacteria" category (probable probiotics), while 49 belong to the "bad bacteria" category (potential pathogens). After PP treatment, PP (100 mg/kg BW and 200 mg/kg BW) can decrease the percentage of potential pathogens in the stool of SD rats. Taken all results together, to consume PP for 28 days can alter the composition of SD rats' gut microbiota. Further analysis is needed to

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explore the effects of changes in SD rats' gut microbiota on relevant mechanisms in the body. The findings of this study can serve as a foundation for the application of PP in regulating gut microbiota as raw materials or products.

Keywords: Papaya Polysaccharides; Rat; Regulation; Intestinal Flora; *In vivo*

1 Introduction

Papaya polysaccharides (PP) may have many advantages have been verified [1-3]. Immunomodulation effects of PP can enhance the function of the immune system, promote the activity of immune cells, improve the body's resistance, and aid in the prevention and alleviation of diseases [4]. PP exhibit significant antioxidant activity. They can neutralize free radicals, reduce cellular oxidative damage, protect cell health, and delay the aging process. PP have certain anti-inflammatory effects. They can alleviate inflammatory reactions and tissue damage, potentially assisting in the treatment of inflammatory diseases. PP can lower blood glucose levels and improve insulin sensitivity, potentially providing auxiliary benefits for blood sugar control in diabetic patients. PP have a protective effect on the liver. They can mitigate liver damage, promote liver cell regeneration, and enhance detoxification functions. Papaya contains an enzyme called papain, which aids in the breakdown of proteins, promoting digestion and absorption of food. Papain can also alleviate heartburn and indigestion caused by excess stomach acid. Papaya is rich in vitamin C, a potent antioxidant that helps protect the body from free radical damage, boosts immunity, and supports the synthesis of collagen, promoting healthy skin, teeth, and gums. In addition, papaya also contains other antioxidants such as vitamin A and vitamin E, as well as trace minerals like magnesium and iron, which are crucial for proper immune system functioning. Certain nutrients found in papaya, such as vitamin C, carotenoids, and fiber, can help lower cholesterol levels, improve cardiovascular health, and reduce the risk of heart disease and stroke. Papaya is rich in vitamin A and carotenoids, which are essential for maintaining healthy eyes. They help prevent eye problems such as night blindness and cataracts, and promote retinal function. The fiber content in papaya helps promote bowel movements, prevent constipation, and maintain a healthy gut. Additionally, papain can assist in the treatment of indigestion and gastric ulcers [5-10].

The importance of gut microbiota composition variations included digestion and nutrient absorption, immune function, metabolic health, mental health, disease risk and prevention, drug metabolism and response etc [11-16]. The gut microbiota plays a crucial role in breaking down complex carbohydrates, fiber, and other components that our bodies cannot digest on their own. It helps in the production of enzymes and metabolites that aid in digestion and nutrient absorption. The gut microbiota interacts with the immune system and helps regulate its development and function. It assists in training the immune system to distinguish between harmful pathogens and harmless antigens, thus playing a key role in immune response and defense against infections. Alterations in the gut microbiota have been associated with metabolic disorders such as obesity, diabetes, and metabolic syndrome. Certain bacteria are involved in the fermentation of dietary fiber, which produces short-chain fatty acids that contribute to metabolic health. The gut-brain axis refers to the bidirectional communication between the gut and the brain. The gut microbiota influences brain function and behavior through various mechanisms, including the production of neurotransmitters and metabolites that can affect mood, cognition, and mental health. Imbalances or dysbiosis in the gut microbiota have been linked to various diseases, including inflammatory bowel disease, irritable bowel syndrome, allergies, autoimmune disorders, and certain types of cancer. Maintaining a diverse and healthy gut microbiota is important for reducing the risk of these conditions. The gut microbiota can influence the metabolism and efficacy of certain medications. It can modify drug compounds, affecting their absorption, bioavailability, and therapeutic effects. Understanding individual variations in gut microbiota can help personalize drug treatments [17-20]. Given these crucial roles, maintaining a diverse and balanced gut microbiota is essential for overall health and well-being. Lifestyle factors, such as diet, stress levels, antibiotic use, and probiotic supplementation, can influence the composition and diversity of the gut microbiota. Ongoing research is aimed at better understanding the complex interactions within the gut microbiota and developing strategies to promote a healthy gut microbial community.

2 Material and methods

2.1 Chemicals and Reagents

Phosphate-buffered saline (PBS; Sigma-Aldrich, Cat. No. P3813), saline (Taiwan Biotech Co., LTD, Cat. No. 100-120-1101), and Zoletil 50 (Virbac, Carros, France), were used in this study.

2.2 Source of PP

PP were provided from Taiwan Seed Improvement and Propagation Station, Council of Agriculture, Taiwan.

2.3 Experimental Animals and Experimental Design

Adult male 18 Sprague Dawley (SD) rats [6 weeks old; body weight (BW) between 151-175 g] with specific pathogen-free conditions were used for this study, were purchased from BioLASCO Taiwan Co., Ltd. (Yilan, Taiwan). The environment was maintained room temperature (24-27 °C) and 60%-70% humidity with a photoperiod of 12-hr light/12-hr dark cycle. The study will begin after a week acclimation. The Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC 111062 approved by the IACUC ethics committee. The male 18 SD rats were divided respectively the normal control group (n = 6) and two dose groups (n = 6 per group) of PP (100 mg/kg BW and 200 mg/kg BW). All SD rats were fed with standard laboratory diet (No. 5053, LabDiet®; PMI Nutrition International, St. Louis, MO, USA) ad libitum during the experimental period. The SD rats in two dose groups of PP were respectively administrated with 100 mg/kg BW and 200 mg/kg BW PP once per day for 4 weeks. SD rats' stool were collected and detected the intestinal flora by using EPI2ME web interface algorithm (Oxford Nanopore Technologies, ONT), CLC Genomics Workbench (ver. 22.0.2; Qiagen), Weighted UniFrac and Bray-Curtis analyses, and PERMANOVA statistical method before and after PP oral administration by gavage.

2.4 Statistical Analysis

SPSS (Statistical package for the social sciences) statistical software (version 28.0) were used for statistical analysis. Measurement data were expressed as mean ± standard deviation (SD). All comparisons were made by one-way ANOVA (Analysis of Variance). All significant differences are reported at $^{*}p < 0.05$.

3 Results and discussion

3.1 Strain Identification

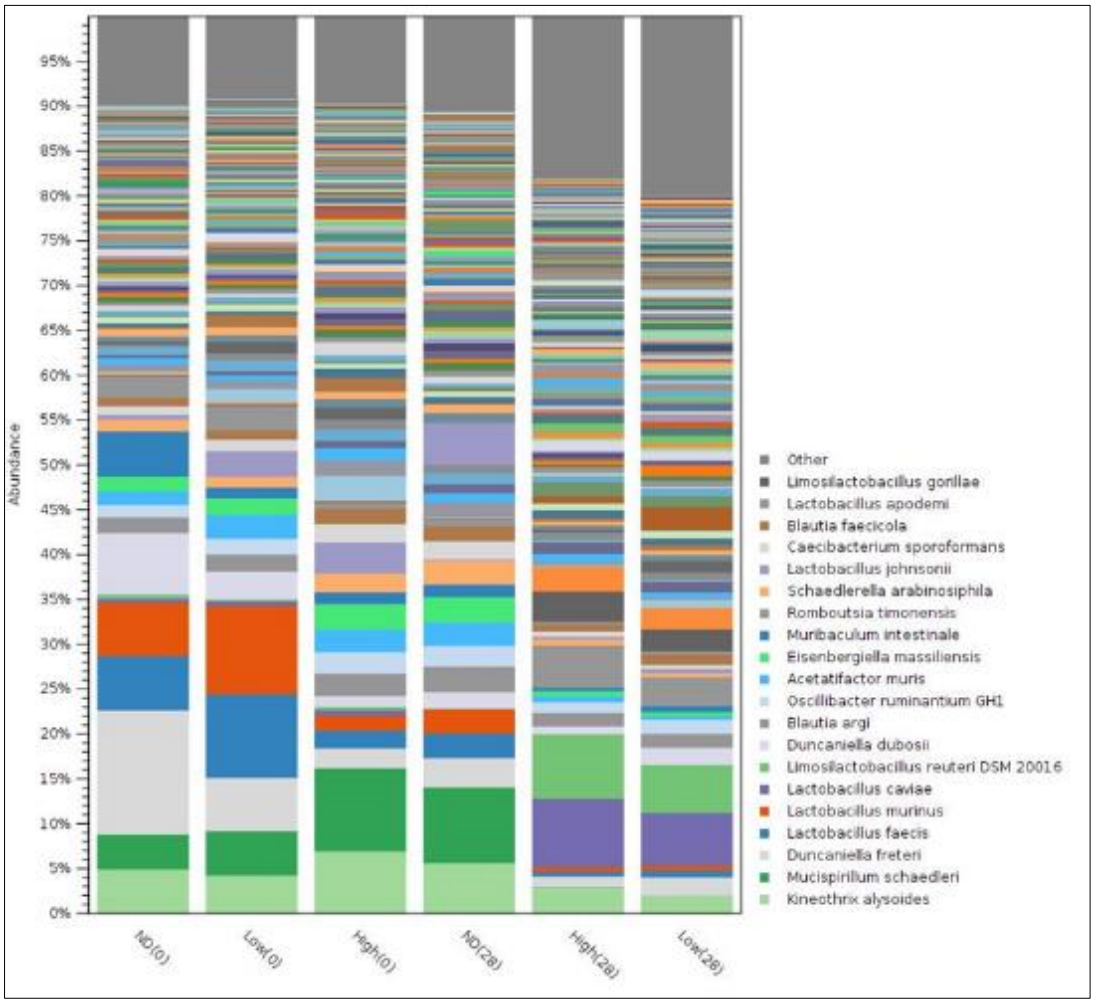
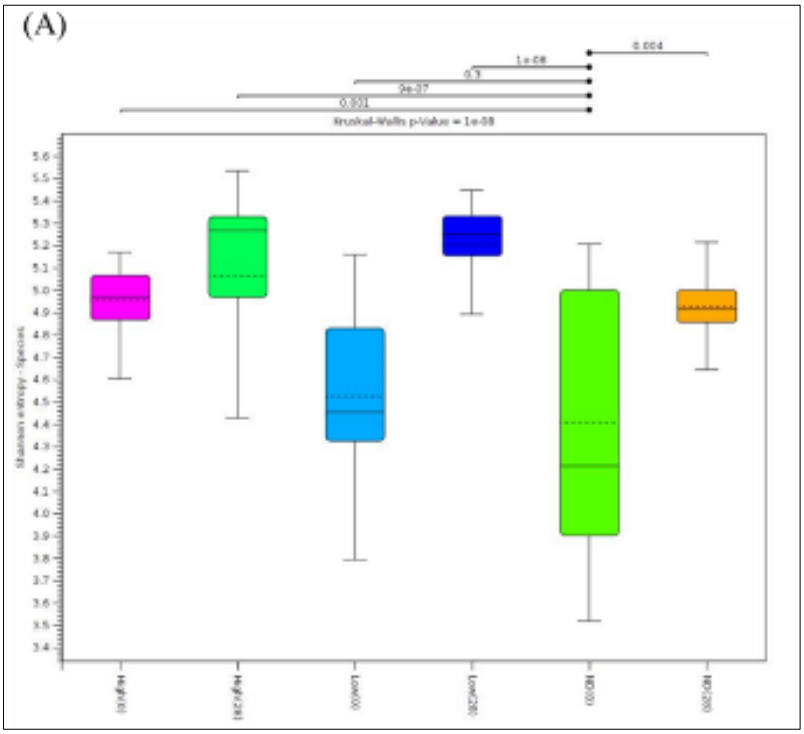
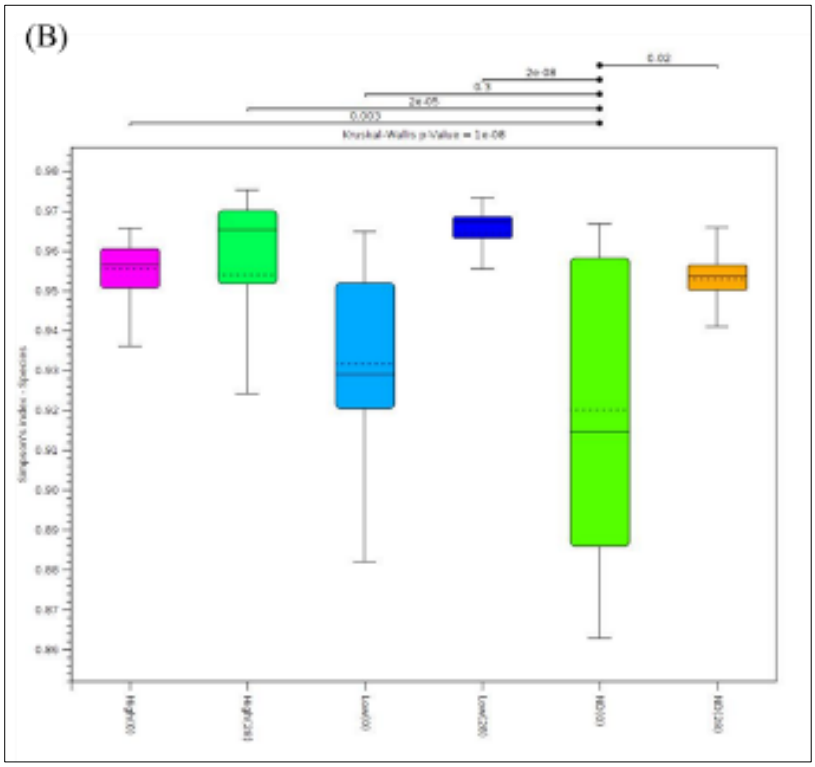


Figure 1 Stacked bar chart of strain identification

Long-read sequencing platform, Oxford Nanopore Technologies (ONT), was used to classify the gut microbiota in rat fecal samples. Whole-genome DNA was extracted from 200 mg of fecal sample using a commercial extraction kit. The SQK-16S024 kit (ONT) was employed to construct libraries from 10 ng of genomic DNA from each fecal sample. Equimolar libraries prepared from 24 fecal samples were loaded onto the MinION MK1C sequencer along with the R9.4.1 flow cell for a 16-hour sequencing run. The resulting sequencing reads were aligned, annotated, and statistically analyzed using the EPI2ME web interface algorithm (ONT) and CLC Genomics Workbench (ver. 22.0.2; Qiagen) [21-23]. A total of 945 bacterial strains were identified through comprehensive strain identification, and the results were visualized using a stacked bar chart representing Operational Taxonomic Units (OTUs) (Figure 1).

3.2 Analysis of Gut Microbiota Abundance, Variability, and Dissimilarity





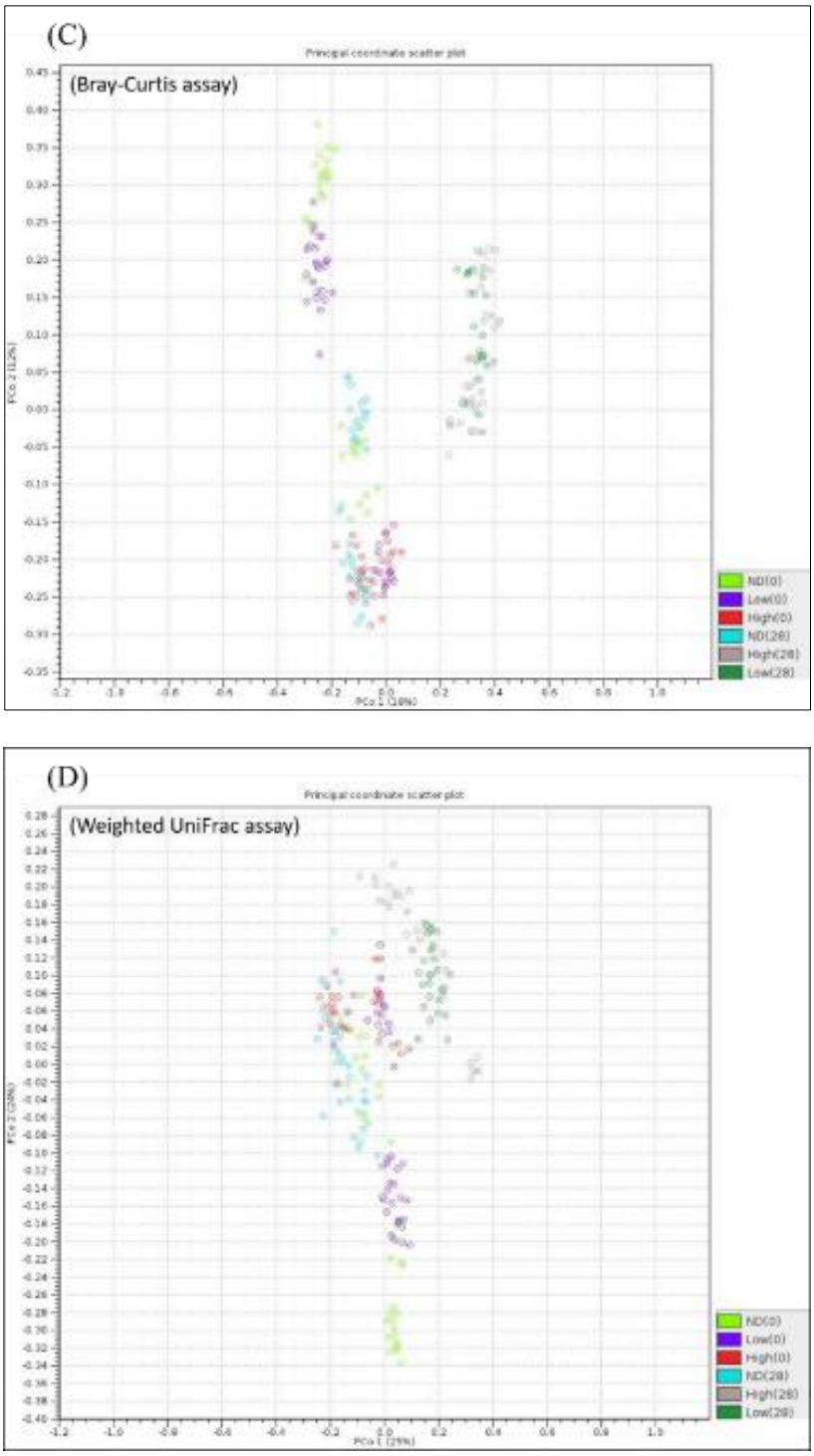


Figure 2 Analysis of gut microbiota abundance, evenness, variability, and dissimilarity. (A) Analysis of the abundance and evenness of identified bacterial species within each group using Shannon entropy. (B) Analysis of the abundance and evenness of identified bacterial species within each group using Simpson index. (C) Analysis of the dissimilarity of identified bacterial species between different groups using Weighted UniFrac. (D) Analysis of the dissimilarity of identified bacterial species between different groups using Bray-Curtis

The obtained sequencing reads were analyzed using the CLC Genomics Workbench (ver. 22.0.2; Qiagen) software for various analyses [20-26]. Firstly, the software executed Shannon entropy and Simpson index calculations to assess the abundance and evenness of identified bacterial species within each group. Additionally, the CLC Genomics Workbench (ver. 22.0.2; Qiagen) software performed Weighted UniFrac and Bray-Curtis analyses to measure the dissimilarity of

identified bacterial species between different groups. Subsequently, the PERMANOVA statistical method was employed to determine the significance of differences in the composition of bacterial species between groups (Figure 2).

3.3 Analysis of Specific Bacterial Species Proportional Changes between Groups

Following the sequence read alignment, annotation, and statistical analysis using CLC Genomics Workbench (ver. 22.0.2; Qiagen), the relative abundance changes of identified bacterial species were compared among different groups, with the normal diet Day 0 group serving as the reference. The statistical significance of the relative abundance changes of identified bacterial species in the fecal samples was determined [27-29].

After comparing the relative abundance changes of identified bacterial species in the fecal samples following 28 days of high-dose PP consumption (200 mg/kg BW) with the normal diet group, the following 45 intestinal bacterial strains showed statistically significant differences: *Lactobacillus caviae*; NR_157747.1, *Limosilactobacillus reuteri* DSM 20016; NR_119069.1, *Duncaniella freteri*; NR_170509.1, *Romboutsia timonensis*; NR_144740.1, *Limosilactobacillus gorillae*; NR_134066.1, *Limosilactobacillus reuteri*; NR_113820.1, *Limosilactobacillus reuteri* DSM 20016; NR_075036.1, *Mucispirillum schaedleri*; NR_042896.1, *Duncaniella dubosii*; NR_170508.1, *Paludicola psychrotolerans*; NR_158111.1, *Lactobacillus murinus*; NR_112689.1, *Lactobacillus faecis*; NR_114391.1, *Muribaculum intestinale*; NR_144616.1, *Intestinimonas butyriciproducens*; NR_118554.1, *Ruminococcus albus* 7 = DSM 20455; NR_115230.1, *Fournierella massiliensis*; NR_156911.1, *Romboutsia ilealis*; NR_125597.1, *Limosilactobacillus oris;* NR_118973.1, *Eubacterium coprostanoligenes*; NR_104907.1, *Paraclostridium benzoelyticum*; NR_148815.1, *Tepidibaculum saccharolyticum*; NR_169458.1, *Lactobacillus apodemi*; NR_112752.1, *Harryflintia acetispora*; NR_152059.1, *Lactobacillus wasatchensis*; NR_147709.1, *Colidextribacter massiliensis*; NR_147375.1, *Clostridium saudiense*; NR_144696.1, *Ihubacter massiliensis*; NR_144749.1, *Gemmiger formicilis*; NR_104846.1, *Lactobacillus nenjiangensis*; NR_125563.1, *Ruminococcus callidus*; NR_029160.1, *Levilactobacillus suantsaiihabitans*; NR_170422.1, *Ruthenibacterium lactatiformans*; NR_151900.1, *Hungateiclostridium saccincola*; NR_152684.1, [*Eubacterium*] *siraeum*; NR_118675.1, *Lachnoclostridium pacaense*; NR_147396.1, *Muricomes intestini*; NR_144617.1, *Abyssicoccus albus*; NR_156906.1, *Neglecta timonensis*; NR_144736.1, *Butyricicoccus faecihominis*; NR_152060.1, *Flavonifractor plautii*; NR_029356.1, *Ruminococcus flavefaciens*; NR_025931.1, *Negativibacillus massiliensis*; NR_147378.1, *Enterorhabdus caecimuris* B7; NR_115868.1, *Anaerotruncus rubiinfantis*; NR_147398.1, *Roseburia hominis* A2-183; NR_074809.1.

Comparing the relative abundance changes of identified bacterial species in the fecal samples after 28 days of low-dose PP consumption (100 mg/kg BW) with the normal diet group, the following 46 intestinal bacterial strains showed statistically significant differences: *Lactobacillus caviae*; NR_157747.1, *Limosilactobacillus reuteri* DSM 20016; NR_119069.1, *Romboutsia timonensis*; NR_144740.1, *Eubacterium coprostanoligenes*; NR_104907.1, *Limosilactobacillus gorillae*; NR_134066.1, *Limosilactobacillus reuteri*; NR_113820.1, *Duncaniella freteri*; NR_170509.1, *Limosilactobacillus reuteri* DSM 20016; NR_075036.1, *Paludicola psychrotolerans*; NR_158111.1, *Lactobacillus murinus*; NR_112689.1, *Mucispirillum schaedleri*; NR_042896.1, *Intestinimonas butyriciproducens*; NR_118554.1, *Acutalibacter muris*; NR_144605.1, *Fournierella massiliensis*; NR_156911.1, *Lactobacillus faecis*; NR_114391.1, *Ruminococcus albus* 7 = DSM 20455; NR_115230.1, *Tepidibaculum saccharolyticum*; NR_169458.1, *Ruthenibacterium lactatiformans*; NR_151900.1, *Lactobacillus intestinalis*; NR_117071.1, *Lactobacillus colini*; NR_156075.1, *Muribaculum intestinale*; NR_144616.1, *Papillibacter cinnamivorans*; NR_025025.1, *Neglecta timonensis*; NR_144736.1, *Lactobacillus rogosae*; NR_104836.1, *Limosilactobacillus oris*; NR_118973.1, *Paraclostridium benzoelyticum*; NR_148815.1, *Lactobacillus gasseri* ATCC 33323 = JCM 1131; NR_075051.2, *Lacticaseibacillus hulanensis*; NR_171466.1, *Frisingicoccus caecimuris*; NR_144612.1, *Cuneatibacter caecimuris*; NR_144608.1, *Microbacterium pseudoresistens*; NR_116843.1, *Romboutsia ilealis*; NR_125597.1, *Bacteroides sartorii* JCM 17136 = DSM 21941; NR_117373.1, *Oscillibacter ruminantium* GH1; NR_118156.1, *Lactobacillus apodemi*; NR_112752.1, *Blautia faecicola*; NR_170495.1, *Lachnoclostridium pacaense*; NR_147396.1, *Colidextribacter massiliensis*; NR_147375.1, *Prevotella koreensis*; NR_165743.1, *Citrobacter cronae*; NR_170426.1, *Lactobacillus nenjiangensis*; NR_125563.1, *Enterorhabdus caecimuris* B7; NR_115868.1, *Monoglobus pectinilyticus*; NR_159227.1, *Lactobacillus hominis* DSM 23910 = CRBIP 24.179; NR_125548.1, *Hungateiclostridium saccincola*; NR_152684.1, *Rodentibacter ratti*; NR_156998.1.

3.4 Creation of Heat Map

Using CLC Genomics Workbench (ver. 22.0.2; Qiagen) software, a heat map was generated (Figure 3) by selecting bacterial strains in the high (28) group that exhibited a relative abundance increase or decrease of more than 4-fold. After database analysis, a total of 945 intestinal bacterial strains were identified in this experiment. It was observed that the proportions of intestinal bacterial communities changed after consuming PP. Among them, 42 bacterial strains showed an increase in abundance after PP consumption, including *Clostridium leptum, Clostridium methylpentosum, Eubacterium siraeum, Abyssicoccus albus, Bombilactobacillus mellis, Butyricicoccus faecihominis, Citrobacter cronae, Clostridium saudiense, Erysipelothrix piscisicarius, Eubacterium coprostanoligenes, Faecalibacterium prausnitzii,*

Frisingicoccus caecimuris, Gemmiger formicilis, Harryflintia acetispora, Hungateiclostridium alkalicellulosi, Hungateiclostridium saccincola, Hydrogenoanaerobacterium saccharovorans, Ihubacter massiliensis, Intestinimonas butyriciproducens, Labilibacter marinus, Lactobacillus animalis, Lactobacillus caviae, Lactobacillus intestinalis, Lactobacillus nenjiangensis, Lactobacillus wasatchensis, Levilactobacillus suantsaiihabitans, Limosilactobacillus gorillae, Limosilactobacillus oris, Limosilactobacillus reuteri, Limosilactobacillus reuteri DSM 20016, Monoglobus pectinilyticus, Negativibacillus massiliensis, Paludicola psychrotolerans, Paraclostridium benzoelyticum, Prevotella colorans, Romboutsia ilealis Romboutsia timonensis Roseburia hominis A2-183, Ruminococcus albus 7 = DSM 20455 Ruminococcus callidus Ruminococcus flavefaciens, Ruthenibacterium lactatiformans etc.

After consuming PP, a total of 12 bacterial strains showed a decrease in abundance. These strains include *Anaerotaenia torta, Butyricimonas phoceensis, Duncaniella dubosii, Duncaniella freteri, Gabonia massiliensis, Gabonibacter massiliensis, Lactobacillus apodemi, Lactobacillus faecis, Lactobacillus murinus, Mucispirillum schaedleri, Muribaculum intestinale, Parabacteroides goldsteinii DSM 19448 = WAL 12034*.

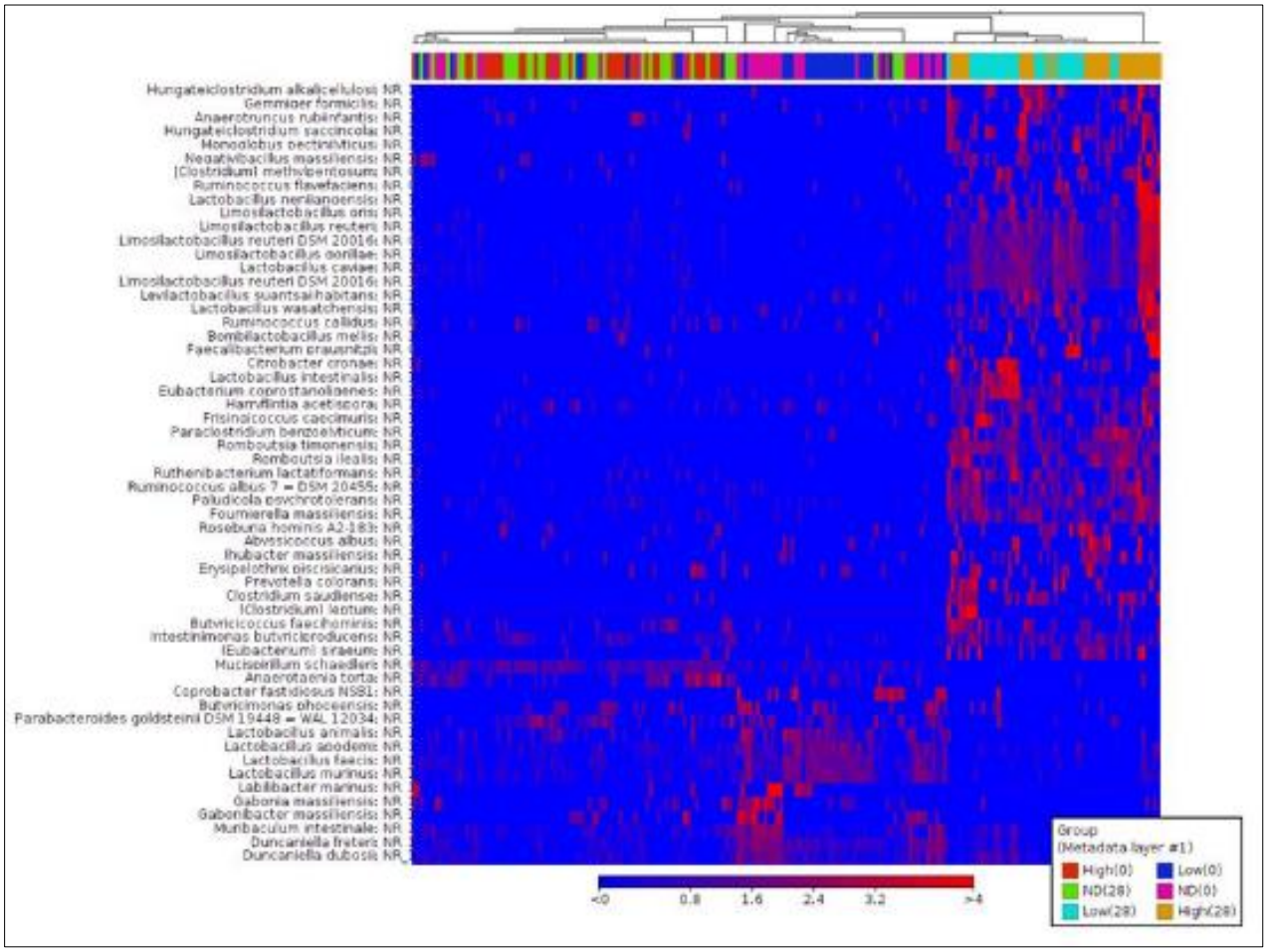


Figure 3 Establishment of heat map after PP treatment in SD rats

3.5 Category of Probable Probiotics and Potential Pathogens after PP Treatment

According to the classification provided by the NIH Office of Dietary Supplements, the commonly recognized probiotic bacterial genera include *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Escherichia*, and *Bacillus*, totaling 6 genera. On the other hand, based on the Virus Pathogen Database and Analysis Resource (ViPR), there are 24 pathogenic bacterial genera, including *Clostridium* and *Helicobacter*. Out of the total 118 bacterial genera considered, 69 belong to the "good bacteria" category (probable probiotics), while 49 belong to the "bad bacteria" category (potential pathogens). To determine the ratio of good to bad bacteria, the relative abundance of each bacterial genus can be analyzed using OTU (Operational Taxonomic Units) data. By calculating the proportion of good bacteria to bad bacteria, you can assess the overall balance between the two categories [26-29]. The results were shown that after PP treatment in SD rats, PP (100 mg/kg BW and 200 mg/kg BW) can significantly decrease the percentage of potential pathogens in the stool ($p < 0.05$) (Figure 4).

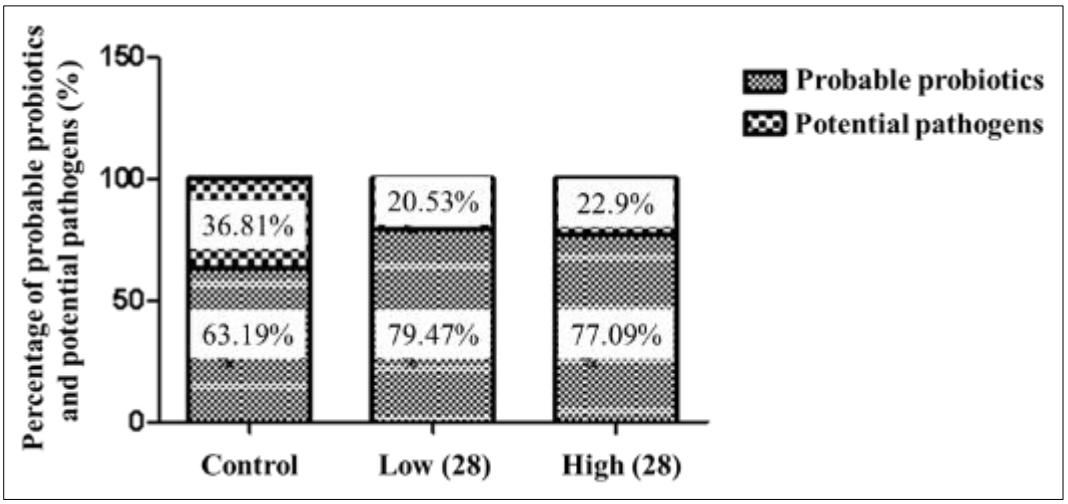


Figure 4 Percentage of probable probiotics and potential pathogens after PP treatment

4 Conclusion

After feeding SD rats with papaya polysaccharide for 28 days and analyzing the gut microbiota using long-read sequencing technology (ONT), a total of 945 intestinal bacterial strains were identified through database analysis. Changes in the abundance and evenness of gut microbiota were observed in the two groups of SD rats fed with PP. Furthermore, based on the analysis of gut microbiota abundance, variability, dissimilarity, and specific bacterial species proportional changes between groups, it can be concluded that there were differences between the experimental groups and the normal control group, but no significant differences were observed between the high and low doses of PP. In summary, the results indicate that consuming PP for 28 days can alter the composition of the gut microbiota. Further analysis is needed to explore the effects of changes in the gut microbiota on relevant mechanisms in the body. The findings of this study can serve as a foundation for the application of PP in regulating gut microbiota as raw materials or products.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

The Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC 111062 approved by the IACUC ethics committee.

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