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Microbial fermentation of blue honeysuckle (*Lonicera caerulea* L.) polyphenol promotes insulin secretion and intervenes in obesity by promoting short-chain fatty acids production

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ABSTRACT

During obesity treatment, clinicians have been burdened by the ever-increasing number of patients and the side effects of drug treatment. Finding new plant-derived active ingredients as an intervention for obesity has become a research focus. In this study, Plackett-Burman and Central Composite Design experiments, plant-wide target metabolomics technology, simulated human colon fermentation test, and cell experiment *in vitro* were successively used to investigate the effects of fermented and unfermented polyphenols (FP and UP, respectively) of blue honeysuckle on obesity. After the optimization of the response surface method, polyphenol content ((1878.08 ± 14.53) mg/100 g) increased by 2.63 times after *Lactobacillus rhamnosus* 6224 and *Saccharomyces cerevisiae* W5 mixed fermentation. There were 23 significantly different metabolites were observed in FP and UP. Of them, 7 different metabolites were significantly and positively correlated with short-chain fatty acids (SCFAs), whereas negatively correlated with lipopolysaccharides (LPS). The contents of these 7 metabolites were significantly increased in FP. Furthermore, the effects of FP and UP on the SCFA biosynthesis pathway were different. Cell experiments revealed that polyphenols can significantly promote the secretion of gastrointestinal endogenous hormones and insulin and inhibit interleukin-6 levels. Linear regression analysis revealed SCFAs had a positive regulatory effect on glycolipid metabolism, whereas LPS had a negative effect. Overall, these crucial findings provide an economical and simple method for obtaining blue honeysuckle polyphenols and laying a theoretical foundation for its application in the therapeutic intervention against obesity.

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1. Introduction

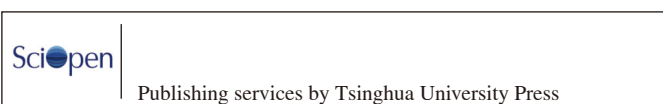
Obesity is a metabolic disease associated with glycolipid metabolism disorder and chronic inflammation. The incidence of obesity is significantly increasing globally^[1]. According to statistics,

more than 600 million adults and approximately 340 million adolescents worldwide are obese. Obesity aggravates the development of other related metabolic diseases (type II diabetes, non-alcoholic fatty liver disease) and poses a huge economic burden on society^[2]. Therefore, preventing and treating obesity is necessary. So far, many therapeutic strategies have been developed for obesity, mainly including drugs (orlistat and sibutramine) and bariatric surgery. However, these strategies are associated with certain risks and side effects^[1,3]. Therefore, a new method to prevent and treat obesity is urgently sort.

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A high-fat diet (HFD) destroys the gut microbiota ecology, reduces short-chain fatty acid (SCFA) production through microbial metabolism, increases lipopolysaccharide (LPS) production, and ultimately disrupts the normal glycolipid metabolism^[4-6]. On the one hand, microbial metabolism produces SCFAs, such as butyric acid, propionic acid, and acetic acid. These SCFAs promote the secretion of glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and cholecystokinin (CCK) by intestinal L cells and increasing satiety, thus reducing food intake and promoting insulin (INS) secretion by islet β cells^[1,7-9]. On the other hand, a decrease in LPS production reduces the secretion of interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α) by mononuclear macrophages, thereby reducing insulin resistance (IR) and alleviating the occurrence of obesity^[1,9-11]. Diet is a crucial factor in determining any alteration in the gut microbiota composition, which then alters microbial metabolites and is a crucial player in the occurrence and development of obesity and related diseases^[3,10,12]. Many bioactive phytochemicals can affect metabolite production by gut microbiota and reduce the obesity risk^[4,13].

Polyphenol is a large class of plant secondary metabolites that contain one or more aromatic rings and 2 or more hydroxyl groups. Polyphenols can isolate or inhibit reactive oxygen and nitrogen, transfer electrons to free radicals, activate antioxidant enzymes, improve oxidative stress and inflammation, and have exhibited good preventive effects against various diseases, such as diabetes, obesity, cancer, and cardiovascular diseases^[14-18]. Polyphenols inhibit fat digestion and absorption by regulating fat transport-related proteins^[13]. Polyphenols have the potential to be used as a functional food, nutritional drug, or pharmaceutical ingredient that can offer a healthy quality of life. Accordingly, the relationship between dietary health and plant bioactive ingredients has become a research hotspot^[12,15,17]. However, significant differences are observed in the composition and function of polyphenols from different sources. Therefore, researchers are striving to identify more polyphenol sources and explore their potential functions.

Lonicera caerulea L., also known as blue honeysuckle (BH), is a perennial bush plant belonging to the Caprifoliaceae family. This plant contains various bioactive compounds. As an emerging fruit, BH has recently attracted considerable attention^[16,19]. Polyphenolic compounds (flavones, flavonoids, phenolic acids, anthocyanins, tannins) are the main active components of BH, with their content being higher in BH than in raspberry, strawberry, blueberry, and blackcurrant^[16,19-20]. Consequently, more people are focusing on this emerging fruit.

Fresh BH have a soft texture and tender skin, are perishable, and have a short storage time, which limits its widespread use^[19]. Meanwhile, the low polyphenol extraction rate is a crucial factor limiting large-scale polyphenol development and application^[21]. Therefore, seeking a simple, green, and pollution-free method to obtain polyphenols through BH processing is critical for expanding its value. Phenolic compounds are present in plants as soluble (aglycans, conjugated glycosides, and soluble esters) and insoluble forms^[22]. Insoluble polyphenols are covalently bound to components that compose the cell wall structure and cannot be directly extracted using organic solvents, and most of them are treated as waste, which greatly reduces polyphenol bioavailability^[17,23].

Insoluble-bound polyphenols can be released through alkaline, acidic, or enzymatic treatment. However, alkaline hydrolysis

breaks ester bonds and acid hydrolysis breaks glycosidic bonds. Furthermore, the final product of enzymatic hydrolysis is usually sugar, which in turn inhibits enzymatic activity^[24]. To overcome these shortcomings, some studies have suggested using microbial fermentation to obtain more soluble polyphenols, as microbes can consume sugars released through enzymatic reactions^[24]. Microbial fermentation is an effective way to promote the biological activity of final products by breaking the bond between phenolics and other substituents in conjugated molecules based on microbial enzymes produced^[25]. Fermentation-induced cell wall structural disruption can release and/or induce the synthesis of various bioactive compounds. Among these compounds, the release of flavonoids in plant foods during fermentation increases the total phenolic content of plant-based materials after fermentation^[26]. Specifically, various changes in phenolic components of plant-based materials can be attributable to microbial depolymerization and degradation of macromolecular polyphenols, as well as the microbial metabolism of phenolic substances^[27]. Microorganisms change the plant substrate composition by hydrolyzing polyphenols and glucosinolates, peptides, secondary metabolites, SCFAs, vitamins, and polysaccharides. This process is mainly mediated by enzymatic reactions carried out by enzymes such as dehydrogenase, reductase, decarboxylase, tannase, cellulases, glucosidase, and esterase^[26-30]. Microorganisms can modify and biotransform phenolic compounds during fermentation to improve substrate bioavailability and bioactivity^[28,31]. Therefore, microbial fermentation has become a new method of obtaining more free polyphenols.

Lactic acid bacteria (LAB) fermentation is among the oldest, most economical, and most valuable biotechnology methods. Most studies on fermented fruit pulp have been conducted using LAB^[21,32]. However, many berries are acidic and inhibit microbial growth, while LAB cannot digest some of their main components, such as fiber and inulin. Bioactive compounds such as polyphenols, which are abundant in fruits, may inhibit microbial growth^[27]. Therefore, microorganisms that can efficiently utilize BH as a substrate and convert it to polyphenols must be screened. Yeast has attracted considerable attention as a fermentation starter culture because of its higher viability in fruits than LAB and its good performance during fermentation^[31,33]. Most berry fermentation strains are currently single, and very few studies have investigated on mixed fermentation of fruit pulp by using LAB and yeast. Therefore, in this study, LAB, yeast, and mixed fermentation methods were used to ferment BH to explore the effect of microbial fermentation on the content and biological activity of BH polyphenols (BHP) and to select the most suitable BHP fermentation method.

The effects of fermentation on the chemical composition and function of plant foods have been partially reported. However, the effects of microbial fermentation on the BHP composition and obesity intervention have not been studied^[24,29]. Therefore, BH, a characteristic small berry in northeast China, was considered the research object in this study. Moreover, microbial fermentation was assumed to improve the free polyphenol content and activity of BH, so that it could better intervene in obesity occurrence. This study 1) screened out strains that could ferment BH from among 17 LAB strains and 3 yeast strains preserved in the experiment; 2) compared the antioxidative and glucolipid metabolism abilities of BHP through single bacterial fermentation, mixed bacterial fermentation, and unfermentation, and

determined the most suitable mode of BHP fermentation; 3) optimized fermentation conditions by using the response surface methodology to increase the free polyphenol content; 4) explored the effect of microbial fermentation on the BHP composition through plant-wide target metabolomics technology, and the key compounds that affected the polyphenol properties were identified; 5) clarified the effects of BHP on SCFA content produced through microbial metabolism and the SCFA synthesis pathway by using SCFA target metabolomics technology; and 6) elucidated the potential mechanism of BHP intervention in obesity at the cellular level. The results can enrich the microbial sources of BH fermentation and provide a theoretical basis for the application of BHP obtained through fermentation to intervene in obesity.

2. Materials and methods

2.1 Plant materials and reagents

BH (Lanjingling, Autumn 2020 harvest) was collected from Harbin, China, and provided by professor Huo Junwei from Northeast Agricultural University (Harbin, China) and Harbin Senmeiyuan Biotechnology Co., Ltd. (Harbin, China). Fully ripe BH was manually picked, stored in a sealed container at -20°C , thawed at room temperature, and mashed in a juicer (L3-C8, Joyoung, China). Then, the homogenate was mixed with deionized water at a 1:1 ratio, sterilized for 20 min at 108°C , and named BH pulp for screening BH fermentation strains^[31,34]. Table S1 presents the main reagents used in the experiment. The reagents used for determining the phenolic composition are all chromatographically pure, and other analytical reagents were purchased from Tianjin Kemio Chemical Reagent Co., Ltd.

2.2 Strain, medium, and culture conditions

The 17 LAB and 3 yeast strains used in the experiment (Table S2) were provided by the Key Laboratory of Microbiology at Heilongjiang University and the China Center of Industrial Culture Collection. MRS and YPD media were used for culturing LAB and yeast, respectively. The Supplementary material presents the medium composition and culture conditions.

2.3 Screening of fermentation strains

2.3.1 Viable count and pH measurement

The plate counting method was used to screen LAB and yeast, which could grow on the BH pulp (pH was approximately 5.0) even in the absence of a carbon source^[26-28,31]. The Supplementary material lists the detailed methods.

2.3.2 Determination of polyphenol content and DPPH radical scavenging ability

To screen LAB and yeast strains with a strong ability to ferment the BH pulp at pH 5.0, 14 strains that could grow using the pulp as a substrate were inoculated with the pulp and cultured at 37°C and 30°C , 140 r/min for 72 h, respectively. Then, 5 mL fermentation broth was collected at 0, 24, 48, and 72 h and centrifuged at 12 000 r/min for 20 min.

After diluting the supernatant 10 times with deionized water, the pH of the supernatant was determined using a pH meter (FE28, Mettler-Toledo, Switzerland)^[35]. The standard curve ($y = 0.0105x + 0.0579$, $R^2 = 0.9992$) was plotted using gallic acid (25–200 $\mu\text{g/mL}$) as the standard substance. The total free polyphenol content was expressed in the gallic acid equivalent in mg/mL ^[14,21,36]. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability was determined according to a previous method^[37-38].

2.4 Extraction and preparation of free phenolic compounds

The strains deemed most suitable for fermenting BH were activated and domesticated, inoculated with the BH pulp at a rate of 5% (V/V), and cultured at 36°C , 140 r/min for 48 h. Among them, the LAB to yeast ratio in the mixed fermentation group was 1:1. Furthermore, all subsequent experiments were conducted using BH without microbial fermentation as a control. The Supplementary material offers detailed methods for extracting free polyphenols^[33,34,37].

2.5 Determination of content and bioactivity of free phenolic compounds

The content of free phenolic compounds was determined using the Folin phenol method; gallic acid was used as the standard substance^[21,36]. In this study, the antioxidant ability of free polyphenols was determined by mainly evaluating DPPH radicals, 2,2'-azino-bis-(3-ethylenzthiazoline-6-sulfonate) (ABTS) cation radicals, hydroxyl radicals ($\cdot\text{OH}$), and nitroso (NO_2^-) scavenging ability and total reducing power. The previous description for specific experimental methods was referred to for this procedure^[26,29]. Using deionized water and vitamin C (VC) as blank and positive controls, absorbance was measured using a microplate reader (Spectra Max iD3, Molecular Devices, USA) at room temperature. For α -amylase and α -glucosidase inhibition, refer to the methods of Kowalski et al.^[7], Zhang et al.^[38], and Liu et al.^[39]. Bile acid binding ability was determined according to previous reports^[40].

2.6 Optimization of fermentation conditions

2.6.1 Plackett-Burman (PB) experiment

Based on the single-factor experiment results, the PB experiment was conducted on 6 factors significantly affecting polyphenol content, namely berry ratio (X_1), temperature (X_2), shaking speed (X_3), strain rate (X_4), inoculum dose (X_5), and time (X_6), with polyphenol content (Y) being used as the response value^[24]. Each factor was represented by high (+1), medium (0), and low (−1) levels, respectively. A total of 15 groups of experiments were conducted. Table S3 presents the coding and actual values of each factor variable, and the design factors and levels of the PB experiment are presented in Table S4^[23].

2.6.2 Central composite design (CCD) experiment

Based on the PB experiment results, polyphenol content (Y) was considered as the response value. This response value was used to conduct a CCD experiment on the significant influencing factors of inoculum dose (X_5), berry ratio (X_1), and strain rate (X_4).

Five levels (1.682, 1, 0, -1, -1.682) were designed. The optimal scheme of three factors was obtained through a model fitting. In total, 20 groups of experiments were conducted. Table S5 presents the design factors and levels of the CCD experiments^[41].

2.6.3 Validation experiment

To verify whether the response surface results were accurate, the activated and domesticated strains were inoculated into the BH pulp. According to the predicted conditions obtained in CCD, 5 repeated experiments were conducted to determine the free polyphenol content and compare it with the predicted values. Table S6 presents the experimental design and results^[42-43].

2.7 Determination of phenolic composition

The compositions of fermented and unfermented polyphenols (FP and UP, respectively) were analyzed using the plant-wide target metabolomics technology. Ultra-high performance liquid chromatography (UPLC; ExionLC AD, AB SCIEX, USA) equipped with an AcQuity UPLC HSS T3 liquid chromatography column (2.1 mm × 100 mm, 1.8 μm, Waters, USA) was used^[7,28-29,34,36,44-45]. The detailed methods were given in the Supplementary material.

2.8 In vitro simulation of salivagastro-intestinal digestion

In vitro digestion simulation can be divided into three stages: simulated saliva digestion (SSD), simulated gastric digestion (SGD), and simulated intestinal digestion (SID). To understand the specific methods in detail, refer to previous descriptions^[46-47]. The supernatant was concentrated in a rotating vacuum evaporator (R213, SENCO, China) at 50 °C and simulated -0.095 MPa after centrifugation at 12 000 r/min for 20 min. Finally, FP and UP formed through salivary-gastrointestinal digestion stimulation *in vitro* were freeze-dried (-80 °C, 0.1 Pa, 72 h) using a freeze-drying machine (SJA-10N-80C, Ningbo Shuangjia, Ningbo, China), then used for subsequent experiments.

2.9 Collection and preparation of fecal samples

Fresh fecal samples of 4 obese participants (body mass index (BMI) > 30, 2 men and 2 women) were collected, thoroughly mixed, and termed as the obese group (obese, O). Similarly, fresh fecal samples were collected from 4 normal weight participants (BMI < 25, 2 men and 2 women), thoroughly mixed, and termed as the normal group (normal, N)^[2]. The 8 stool providers were all aged 22–26 years, in good health, and provided informed consent. They had no smoking and drinking habits; had no diabetes, kidney disease, liver disease, or other intestinal diseases; and had not taken antibiotics in the 3 months before study participation. The work described here was performed according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) for human experimentation. The Ethics Committee of Qiqihar Medical College reviewed this study on *in vitro* anaerobic fermentation in the simulated human colon. Based on the results, the committee considered that the trade-off between the risks and expected benefits of this study was reasonable and consistent with ethical principles.

The fecal samples were mixed with sterile phosphate buffer (pH 7.4, 0.1 mol/L) at a 1:3 (*m/V*) ratio and filtered with sterile gauze to remove impurities. The obtained fecal homogenates were used for inoculation^[48-49]. The experiment was divided into 8 groups: namely normal negative control (NNC), normal positive control (NPC), normal unfermented polyphenol digestion (NUPD), normal fermented polyphenol digestion (NFPD), obese negative control (ONC), obese positive control (OPC), obese unfermented polyphenol digestion (OUPD), and obese fermented polyphenol digestion (OFPD). A negative control was without a sample, and a positive control included fructooligosaccharide (FOS, 10 mg/mL). FP and UP concentrations were both 10 mg/mL.

2.10 In vitro simulated colonic anaerobic fermentation

In vitro fecal fermentation of BHP was conducted using a previously reported method with slight modifications^[31,48,50]. The Supplementary material lists the detailed methods. The LPS, H₂S, and growth hormone (GH) content were determined using the kit, and the specific operation method was performed according to the instructions.

2.11 Determination of SCFAs content

SCFAs were determined through gas chromatography (Clarus680, PerkinElmer, USA) with slight modifications to the original method^[47,49]. The detailed methods are presented in the Supplementary material.

2.12 Cell culture and viability determination

The cell source and culture conditions are given in the Supplementary material. The cell proliferation and toxicity detection kit CCK-8 was used to detect cytotoxicity. The samples (NNC, NPC, NUPD, NFPD, ONC, OPC, OUPD, and OFPD) obtained after *in vitro* fermentation were centrifuged at 4 °C at 12 000 r/min for 20 min and passed through a 0.22-μm sterile filter. The test was divided into 10 groups: NNC, NPC, NUPD, NFPD, ONC, OPC, OUPD, OFPD, UP, and FP.

2.13 Detection of key factors of glycolipid metabolism

The GLP-1, PYY, CCK, INS, IL-6, and TNF-α content was determined using commercial kits^[8,51-53]. The detailed methods are given in the Supplementary material.

2.14 16S rRNA high-throughput sequencing

16S rRNA high-throughput sequencing was conducted using the samples (NNC, NPC, NUPD, NFPD, ONC, OPC, OUPD, and OFPD) of fecal fermentation for 24 h *in vitro*, and data obtained were analyzed on the online platform Majorbio Cloud Platform (www.majorbio.com). Species annotation and clustering were performed for effective OTUs (> 97%). By referring to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the Tax4Fun function was used to predict key enzyme activities in the SCFA metabolism process in different treatment groups, and the potential SCFA synthesis pathway was constructed.

2.15 Statistical analysis

All experiments were conducted independently 3 times. SPSS software (25.0) was used for data analysis. ANOVA was used to analyze the data statistically, and Duncan's test was performed for further statistical analysis. The results were expressed as mean $\pm$ standard deviation (SD). The response surface experiment was designed using Design Expert 10, and SIMCA 14.1 was used to screen differential metabolites. TBtools (1.098745) was used for the heatmap analysis of bioactive components and bioactivity, with Origin 2022b being used for plotting.

3. Results

3.1 Screening of fermentation strains

Microbial fermentation can convert bound polyphenols into free polyphenols in fruits and change the polyphenol composition of the substrate^[28]. Therefore, in this study, 17 LAB and 3 yeast were used to ferment the BH pulp. Then, 11 LAB and 3 yeast strains that could grow on the BH pulp in the absence of any carbon sources were screened using the plate counting method (Fig. S1A, Table S2). Additionally, to screen LAB (11) and yeast (3) strains with a strong ability to ferment the BH pulp at pH 5.0, the total free polyphenol content and DPPH radical scavenging ability were measured at 0, 24, 48, and 72 h (Fig. 1A). The total free polyphenol content and DPPH radical scavenging ability of *Lactobacillus rhamnosus* 6224 were relatively high at 24 (5.25 ± 0.89) mg/mL and (92.74 ± 2.45)%, respectively) and 48 h (5.28 ± 0.71) mg/mL and (94.56 ± 1.96)%. In addition, polyphenol content significantly increased after *L. rhamnosus* 6224 fermentation. Compared with 0 h (4.87 ± 0.73) mg/mL, the polyphenol content at 48 h increased by 8.42% ($P < 0.05$). Compared with *Saccharomyces cerevisiae* 1946 and 32788, *S. cerevisiae* W5 exhibited higher antioxidant ability. Meanwhile, *S. cerevisiae* W5 fermentation could maintain a high pH of BH pulp, whereas *L. rhamnosus* 6224 fermentation decreased the pH of the BH pulp by 15.16% compared with the initial pH (Fig. S1B). Therefore, subsequent experiments were conducted using *L. rhamnosus* 6224 and *S. cerevisiae* W5.

To obtain a higher BHP yield and activity, single and mixed bacterial fermentation methods were used to ferment BH. The free polyphenols were extracted after fermentation, and BHP content was determined. The antioxidant and glycolipid metabolism abilities of BHP *in vitro* were evaluated (Figs. 1B-H and S1C-E). The BHP content of *L. rhamnosus* 6224 and *S. cerevisiae* W5 mixed fermentation (LSF, 1634.79 ± 75.55) mg/mL) was significantly higher than that of the other groups ($P < 0.05$) and was 1.40 times that of the UF group (Fig. 1B). The results of *in vitro* antioxidant ability revealed that the DPPH radical (88.62 ± 0.46)% and ABTS cation radical scavenging abilities (78.57 ± 1.44)% of LSF were comparatively high at a polyphenol concentration of 1.0 mg/mL, and increased in a dose-dependent manner (Figs. 1C and D). Moreover, at a polyphenol concentration of 1.0 mg/mL, the $\cdot\text{OH}$ scavenging ability (approximately 100%) reached the maximum, and the NO_2^- scavenging abilities of UF (98.66 ± 0.30)%, LF (*L. rhamnosus* 6224 fermentation, 99.24 ± 0.17)%, and LSF ($96.49\% \pm 0.04$)% were higher, whereas the reducing power of LSF was relatively weak (1.97 ± 0.04)% (Figs. S1C-E).

α -Amylase and α -glucosidase can decompose starch and glycogen, and glucose absorption is delayed with the inhibition of their enzyme activities, which then controls obesity. Natural phenolic compounds can non-specifically inhibit α -amylase and α -glucosidase activities^[7,54]. Therefore, to preliminarily evaluate whether BHPs can intervene in obesity, their ability to inhibit α -amylase and α -glucosidase activities was measured *in vitro* (Figs. 1E and F). At 4.0 mg/mL polyphenol, LSF-mediated inhibition of α -amylase activity (53.02 ± 3.55)% was the highest, which increased by 59.08% compared to UF and in a dose-dependent manner (Fig. 1E). However, at 4.0 mg/mL polyphenol, UF-, SF- (*S. cerevisiae* W5 fermentation), and LSF-mediated inhibition of α -glucosidase activity exhibited no significant difference ($P > 0.05$), but it was higher than LF (95.60 ± 0.98)% and increased in a dose-dependent manner (Fig. 1F). The binding ability of sodium taurocholate in SF and LSF was positively correlated with the polyphenol concentration, whereas UF and LF exhibited a trend of first increase and then decrease with an increase in the polyphenol concentration (Fig. 1G). At 2.0 mg/mL polyphenol, compared with UF, LF, and SF, the binding ability of sodium taurocholate in LSF (48.48 ± 0.01)% increased by 58.22%, 138.70%, and 12.01%, respectively. The changing trend of the binding ability of sodium glycyrrholate and sodium taurocholate was similar. At 2.0 mg/mL polyphenol, compared with UF and LF, the binding ability of sodium glycyrrholate in LSF (47.84 ± 0.01)% increased by 79.98% and 173.68%, respectively (Fig. 1H).

In summary, combined with polyphenol content, and antioxidant and glucolipid metabolic abilities *in vitro*, the mixed fermentation of BH by *L. rhamnosus* 6224 and *S. cerevisiae* W5 resulted in the highest polyphenol content and activity, indicating that mixed fermentation can be used for subsequent experiments.

3.2 Optimization of fermentation conditions

3.2.1 PB experiment

To obtain more BHP, the PB experiment was conducted to screen out factors that significantly influence BHP content based on single-factor experiments. In the PB experiment, the BHP content increased from (1098.80 ± 61.75) to (1620.21 ± 65.38) mg/100 g, which indicated that fermentation conditions critically affected the BHP content (Table S4). Based on the PB experimental results, the model fitting Eq. (1) represents the relationship of each variable with BHP content. Y (mg/100 g) represents the predicted BHP content, and X represents the variable coding value.

$$Y = 1297.22 + 92.25X_1 + 1.07X_2 - 15.62X_3 + 54.15X_4 + 108.95X_5 + 16.48X_6 \quad (1)$$

Table S7 presents the statistical analysis results of the PB experiment. A P -value was used to determine whether variables significantly affect BHP content. According to the analysis of ANOVA, a statistical difference was observed between regression models ($P = 0.0141$), and an F -test was used to determine the significance of the model equation. R^2 was 0.9163 and adjusted R^2 was 0.8159, indicating that this equation can estimate 91.63% of the variation. The coefficient of variation was relatively low at 5.57%, which indicated the high accuracy and reliability of the experimental result. The precision $9.889 > 4$ indicates that the model can fit the

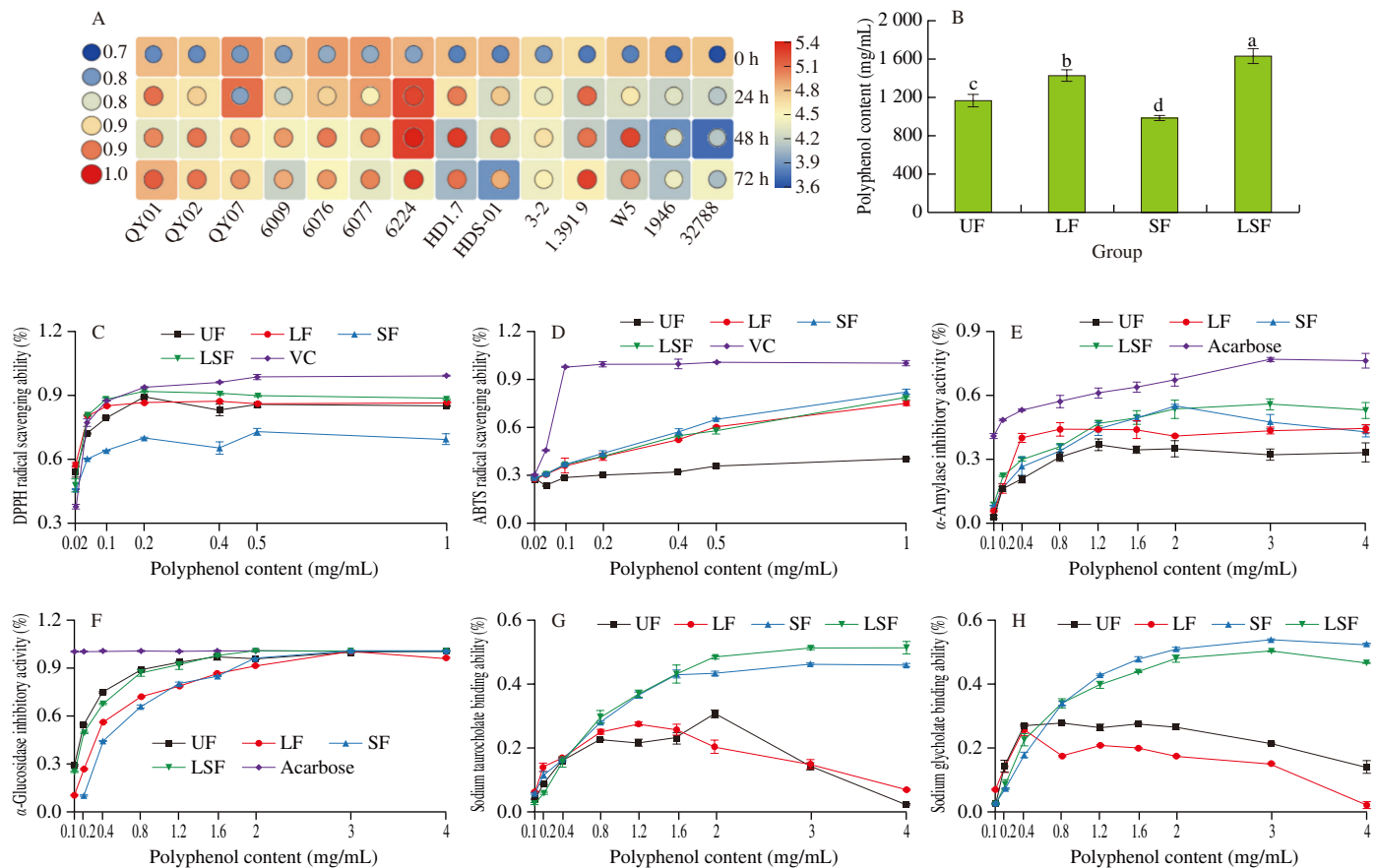


Fig. 1 (A) Total free polyphenol content and DPPH radical scavenging abilities of BH fermented by 11 LAB and 3 yeast strains. The squares represent total free polyphenol content and the circles represent DPPH radical-scavenging abilities. (B) The total free polyphenol content; (C) DPPH radical scavenging ability; (D) ABTS cation scavenging ability; (E) α -amylase inhibition; (F) α -glucosidase inhibition; (G) sodium taurocholate-binding ability; and (H) sodium glycolate binding ability of UF, LF, SF, LSF. Significant differences are indicated with different lowercase letters on the bars ($P < 0.05$).

experimental data well. Moreover, Table S7 shows that the berry ratio (X_1), strain rate (X_4), and inoculum dose (X_5) significantly and positively affect the BHP content.

3.2.2 CCD experiment

Based on the PB experiment, the CCD experiment was conducted to determine the optimal level of factors (X_1 , X_4 , and X_5) that significantly affect the BHP content. Table S8 presents the results of the CCD regression analysis. Figs. S2A-F presents the effects of 3 factors and their interaction on the BHP content of *S. cerevisiae* W5 and *L. rhamnosus* 6224 mixed fermentation. According to the regression analysis results (Table S8), the CCD model terms were extremely significant ($P = 0.0007$), R^2 was 0.8989, and adjusted R^2 was 0.8079. This indicated a good correlation with the model, and 89.89% of the experimental data variability could be explained through this regression model. The precision $10.832 > 4$ indicates that the effective signal-to-noise ratio was appropriate, and the model can better fit the experimental data well. Models X_5X_1 , X_5X_4 , and X_1X_4 all reached a significant level ($P < 0.05$), indicating that the berry ratio, strain rate, and inoculum dose significantly affected the BHP content. When the variable is at the coding level, a quadratic Eq. (2) can be obtained by fitting the experimental results in Table S8. Y (mg/100 g) represents the predicted BHP content, and X represents the variable coding value.

$$Y = 1870.81 - 30.84X_5 + 6.29X_1 - 36.91X_4 + 88.44X_5X_1 + 94.61X_5X_4 - 121.68X_1X_4 - 69.26X_5^2 - 30.55X_1^2 - 56.28X_4^2 \quad (2)$$

In the equation, the coefficients of the quadratic terms are all negative, which highlighted that the surface fitted by the equation had a downward opening and the equation had a maximum value. When the X_5 , X_4 , and X_1 coding levels were 0.458, 0.590, and -0.113, respectively, which means the inoculum dose, berry ratio, and strain rate were 4.917%, 0.449, and 0.361, respectively, the equation had a maximum value, and the predicted BHP content was 1864.704 mg/100 g.

3.2.3 Validation experiment

The maximum value of each variable in the CCD model was chosen to conduct 5 groups of model verification experiments. Table S6 presents the experimental design and results. The t -test comparing the experimental and predicted values revealed that the P -value was 0.4837. This indicated no significant difference between the experimental and predicted values, that is, the CCD optimization model had good fitting performance and provided reliable results. By optimizing the conditions of *L. rhamnosus* 6224 and *S. cerevisiae* W5 mixed fermentation (berry ratio was 1:2.2, the temperature was 34 °C, shaking speed was 160 r/min, *S. cerevisiae* W5 to *L. rhamnosus* 6224 ratio was 1:2.8, inoculum dose was 5%, time was 42 h), the BHP

content reached ($1\,878.08 \pm 14.53$) mg/100 g, 2.63 times of the initial BHP content ((712.88 ± 7.75) mg/100 g). Thus, the BHP content significantly increased in the response surface experiment.

3.3 Effects of microbial fermentation on the BHP composition

Microorganisms may perform phenolic compound biotransformation during fermentation, thereby ensuring they have stronger biological activities. Therefore, to better understand the regulatory effect of BHPs on obesity, how fermentation affects the phenolic substance composition in BH should first be clarified^[19,31]. This study used a plant-wide target metabolomics technology to determine the free polyphenol composition before and after fermentation. Fig. S3 presents the composition chromatograms (total ion current chromatogram) of FP and UP. In total, 683 metabolites were detected, which were majorly divided into 16 categories (Fig. 2A). The relative proportion (RP) of various metabolites in UP and FP was as follows: steroids (0.18%, 0.29%), coumarins (0.26%, 0.30%), phenylpropanoids (0.26%, 0.41%), vitamins (0.31%, 6.29%), lignans (0.73%, 0.45%), terpenoids (2.95%, 3.35%), alkaloids (4.24%, 3.88%), flavones (4.11%, 6.16%), phenols (4.77%, 5.15%), nucleotides (5.78%, 13.52%), lipids (9.32%, 7.80%), others (10.13%, 5.87%), carboxylic acid (11.69%, 8.32%), amino acids (12.74%, 9.72%), carbohydrates (15.19%, 12.06%), and flavonoids (17.35%, 16.43%).

Furthermore, BH contained 224 phenolic metabolites, with RP of 27.48% and 28.90% in UP and FP, respectively. Among those metabolites, rutin (26.48%, 34.29%), cynaroside (21.56%, 2.48%), octopamine (9.93%, 13.84%), cyanidin 3-*O*-rutinoside (3.61%, 3.61%), delphinidin-3-*O*-glucoside (3.43%, 3.61%), quercetin (3.43%, 3.61%),

procyanidin B2 (2.89%, 2.94%), tricetin (2.13%, 6.53%), and epicatechin (1.33%, 2.16%) were accountable for a higher RP of phenolic compounds in UP and FP, respectively. We analyzed the differential metabolites before and after fermentation and selected 23 metabolites with significant differences (variable importance in the projection (VIP) > 1) and measured their RP. Mixed fermentation significantly increased the RP of 13 metabolites, whereas decreased the RP of 10 metabolites (Fig. 2B). Additionally, the RP of rutin (43.71%) and octopamine (17.64%) among the FP differential metabolites was relatively high. The RP of rutin (32.97%) and cynaroside (26.84%) among the UP differential metabolites were also relatively high (Fig. 2C). The BHP composition changed significantly after mixed bacterial fermentation.

3.4 Effects of BHPs on microbial metabolites

Fecal pH is closely related to colon health and is an indirect indicator of bacterial fermentation and metabolites^[47]. Therefore, the pH values of the supernatant at different times of *in vitro* fecal fermentation were determined. pH decreased significantly during the initial fermentation stage, but no significant change was noted after 12 h (Fig. S4A). At 24 h, the pH values of NFPD (5.25 ± 0.02) and OFPD (5.61 ± 0.02) were 3.14% and 2.60% lower than those of NUPD and OUPD, respectively. Additionally, the pH values of the treatment groups decreased significantly compared with those of the NNC and ONC groups, which is consistent with the changing trend noted by Pei et al.^[46].

SCFAs are the main metabolite of gut microbiota fermentation. They play a positive role in the host's health and are the energy source for colon cells^[47,55], with functions such as regulating glycolipid metabolism, resisting pathogens and inflammation, regulating the

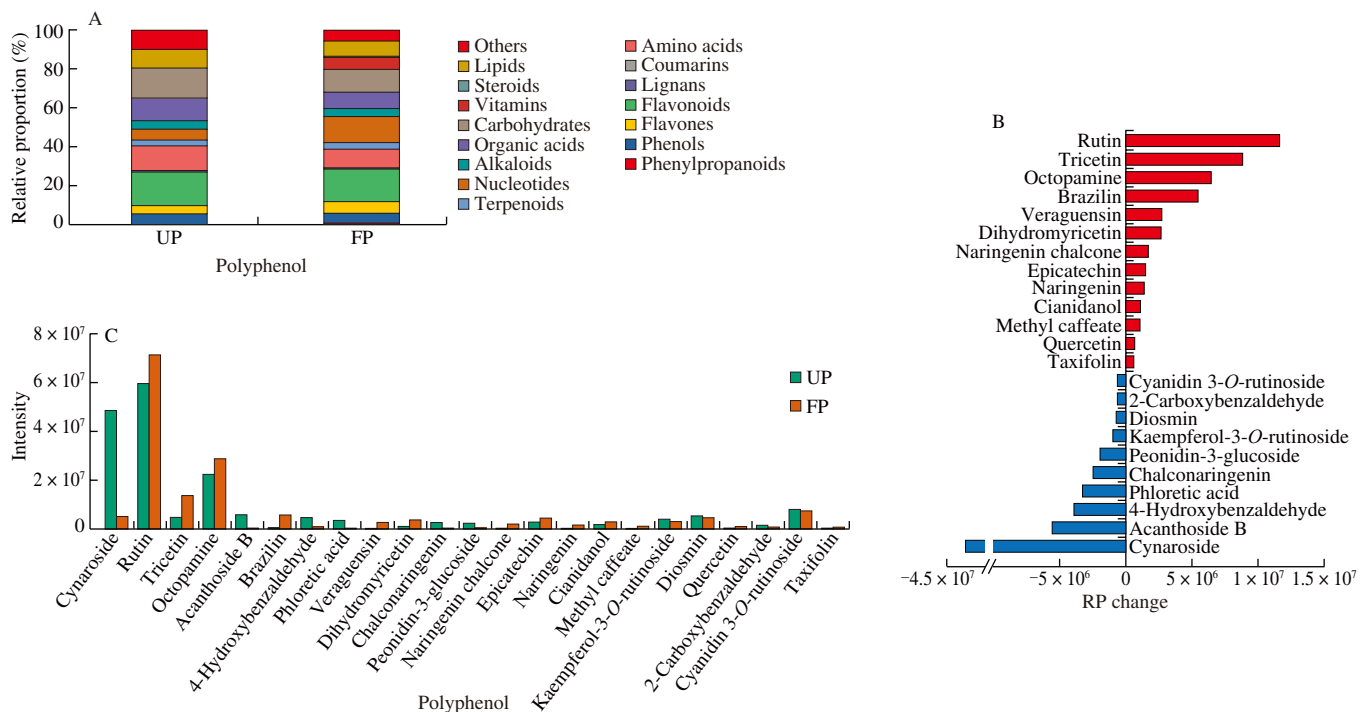


Fig. 2 (A) Composition of UP and FP in BH. (B) Changes in the RP of the significantly different substances in FP compared with those in UP. (C) The RP of significantly different polyphenols in FP and UP.

gut microbiota balance, and improving intestinal function^[56]. Natural polyphenols can affect SCFA generation by interfering with gut microbiota activities^[47]. Therefore, to determine the influence of BHPs on gut microbiota metabolites, SCFA target metabolomics technology was used here to determine the SCFA composition and content at different fermentation times. In total, 6 SCFAs (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid) were detected (Fig. 3A-G). Acetic acid is the main metabolite of most bacteria. It can serve as an energy source for the gut microbiota and peripheral tissues and is a crucial SCFA component^[48]. The acetic acid content in NFPD and OFPD first increased and then decreased as the fermentation time was extended. The content reached the maximum value at 24 h. Polyphenols had a stronger ability to produce acetic acid in group N than in group O (Fig. 3A). Additionally, the acetic acid content was 97.40% higher in NFPD ((3.04 ± 0.29) mmol/L) than in NUPD after 24 h. The acetic acid content was 4.39 times higher in OFPD ((1.80 ± 0.11) mmol/L) than in OUPD. This suggested that the promotion effect of FP on microbial metabolism to produce acetic acid was significantly higher than that of UP.

Propionic acid is an energy source for colon cells, reduces serum cholesterol levels, and inhibits cholesterol and fat synthesis as well as gluconeogenesis, thereby preventing diet-related obesity^[31,48]. The trend of propionic acid content with fermentation time was similar to that of acetic acid. The propionic acid content was 46.07% higher in NFPD ((10.59 ± 0.53) mmol/L) than in NUPD. While the propionic acid content in OFPD ((5.42 ± 0.35) mmol/L) was 28.74% higher than that in OUPD at 24 h. This indicated that the promotion effect of FP on microbial metabolism to produce propionic acid was significantly higher than that of UP (Fig. 4B). In addition, a significant difference in propionic acid was observed between NUPD and OUPD at 24 h ($P < 0.05$) and between NFPD and OFPD ($P < 0.05$). This revealed that BHPs differed significantly in their intervention abilities between N and O; the intervention abilities were stronger in group N.

The isobutyric acid content in group N, except for NNC, was maximum at 24 h, while that in group O, except for OPC, was maximum at 48 h (Fig. 3C). Additionally, the NFPD group ((0.10 ± 0.00) mmol/L) exhibited a 25% increase in the isobutyric acid content compared with the NUPD group at 24 h. By contrast, the OFPD group ((0.04 ± 0.00) mmol/L) exhibited a 33.33% decrease in the isobutyric acid content compared with the OUPD group at 48 h. The promoting effect of FP on microbial metabolism for producing isobutyric acid was significantly higher than that of UP. Butyrate is the main energy source for colon cell metabolism. It also prevents LPS-induced intestinal barrier damage by promoting the expression of the tight junction protein, stimulates IL-18-mediated activation of immunosuppression in combination with GPR109A, helps maintain intestinal homeostasis, and acts as a histone deacetylase inhibitor with anti-inflammatory functions^[1,4]. Different from isobutyric acid, polyphenols possess a stronger ability to promote butyric acid production metabolically in group O. The butyric acid content in OUPD ((4.02 ± 0.17) mmol/L), OFPD ((4.48 ± 0.00) mmol/L), NUPD ((2.12 ± 0.40) mmol/L), and NFPD ((1.74 ± 0.23) mmol/L) were maximum at 48 h (Fig. 3D). Furthermore, the butyric acid content in NUPD and OUPD was significantly different at 48 h ($P < 0.01$), and

NFPD was significantly different from OFPD in butyric acid content ($P < 0.0001$). This indicated that the intervention ability of BHP differed significantly between group N and O. The intervention ability was stronger in group O, meanwhile, the effect of FP was better than that of UP.

The trend of isovaleric acid was similar to that of isobutyric acid. However, the isovaleric acid content of ONC (0.29 ± 0.01 mmol/L) and NNC (0.27 ± 0.02 mmol/L) was the highest at 48 h (Fig. 3E). Valeric acid also offers health benefits, regulating glycolipid metabolism in adipocytes, but its content is relatively low and has gained less attention^[25]. The promoting effects of OUPD ((0.89 ± 0.08) mmol/L) and NUPD ((0.58 ± 0.06) mmol/L) on microbial metabolism-mediated valeric acid production were 74.51% and 176.19% higher than those of OFPD and NFPD at 48 h, respectively (Fig. 3F). Furthermore, as the fermentation time extended, the total acid content first increased and then tended to be stable. NFPD ((15.82 ± 0.54) mmol/L), NUPD ((10.81 ± 0.73) mmol/L), OFPD ((13.40 ± 0.82) mmol/L), and OUPD ((12.16 ± 0.60) mmol/L) reached their maximum total acid content at 24 and 48 h, respectively. Among them, the total acid content was 46.35% higher in NFPD than in NUPD. The total acid content was 10.20% higher in OFPD than in OUPD (Fig. 3G). This indicated that FP exerted a significantly higher promoting effect on microbial metabolism for producing SCFAs than UP.

In addition, on analyzing the RP of SCFAs in each group, we noted that propionic acid had the highest RP, followed by butyric acid, acetic acid, and valerate (Figs. 3H and S4B). With an extension of the fermentation time, the RP of propionic acid gradually decreased. Compared with NNC and ONC, acetic acid content increased in the experimental group, whereas valerate and isovalerate content decreased. Butyric acid content was relatively higher in group O than in group N. NNC and ONC was primarily formed of propionic acid, valeric acid, butyric acid, and isovaleric acid. At 24 h, propionic acid, acetic acid, and butyric acid RPs in NUPD, NFPD, OUPD, and OFPD were 67.13%, 66.96%, and 53.38%; 50.26%, 14.26%, and 19.21%; 5.16%, 16.71%, and 14.60%; and 10.75%, 34.64%, and 28.82%, respectively. These results revealed that SCFAs in each group after BHP intervention were mainly composed of propionic acid, acetic acid, and butyric acid at a ratio of approximately 60:20:20.

To better explain the effects of BHP on SCFA production by gut microbiota, a heatmap was drawn using the SCFA content at different time points, and cluster analysis was performed (Fig. S4C). It was mainly divided into 4 major categories. Among them, changes in SCFAs of NPC and NFPD in the late fermentation period, and those of OPC, OUPD and OFPD, NNC and ONC, and NUPD and NFPD in the early fermentation period exhibited similar trends. This indicated that polyphenols had certain effects on gut microbiota-mediated metabolic production of SCFAs. In addition, the principal component analysis (PCA) was conducted to analyze differences in SCFAs among different treatment groups, as shown in Fig. 3I. The distance between OPC, OUPD, and OFPD was closer, but far away ONC, indicating that BHP addition can change the original SCFA composition in group O, and the effect of SCFAs on gut microbiota metabolism was similar to that of positive control. Meanwhile, OUPD

and OFPD were located on both OPC sides, indicating that certain differences in the SCFA composition existed between OUPD and OFPD. Moreover, NNC was close to NUPD and NFPD and far away from OPC, indicating that BHP affected SCFAs production through gut microbiota metabolism in group N, but the effect was lower than that in the positive control group. NUPD and NFPD were closer, and compared with NUPD, NFPD was closer to NPC. This indicated that the effect of FP on the SCFA composition was similar to that of positive control in group N.

In addition, to investigate the differences in the SCFA composition among different groups, orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed. Among them, the permutation test is a random sorting method used to evaluate whether the OPLS-DA model is accurate. In this study, the R^2 intercept value was 0.027 1, significantly less than the explanatory degree of model variables and less than 0.3. The Q^2 intercept value was -0.074 6, significantly less than the predictive degree of model variables and > 0.05 . It indicated that the model had passed the permutation test. The model does not have to overfit, and subsequent analysis was effective (Fig. S4D). Fig. 3J presents the OPLS-DA results. SCFAs of the 8 treatment groups exhibited a dispersed state. NNC and ONC, NPC and OPC,

NUPD and NFPD, and OUPD and OFPD were relatively close. N and O were grouped into 2 groups. Furthermore, propionic acid and butyric acid, which contribute more to the difference between the groups, were screened out with VIP > 1 (Fig. S4E).

In addition to producing beneficial SCFAs, gut microbiota metabolism produces some harmful LPS. This leads to an increase in the plasma endotoxin content and serious reactions such as increased body fat, insulin resistance, and the expression of pro-inflammatory biomarkers^[10-11]. Furthermore, LPS inhibits INS secretion and damages pancreatic β cells^[9]. To explore the effect of BHP on harmful metabolites, the LPS in the fecal supernatant after fermentation was measured. Compared with NUPD ((3.11 ± 0.12) pg/mL) at 24 h, NFPD ((1.54 ± 0.14) pg/mL) significantly reduced the LPS content. LPS content in NUPD and NFPD was 21.86% and 61.31% lower than in NNC ((3.98 ± 0.38) pg/mL), respectively (Fig. 3K). The inhibitory effect of OUPD ((2.36 ± 0.09) pg/mL) and OFPD ((2.31 ± 0.01) pg/mL) on LPS exhibited no significant difference, which was 31.99% and 33.43% lower than that of ONC ((3.47 ± 0.19) pg/mL), respectively. Meanwhile, GH can affect lipid metabolism, and FP addition significantly affects GH content (Fig. 3L).

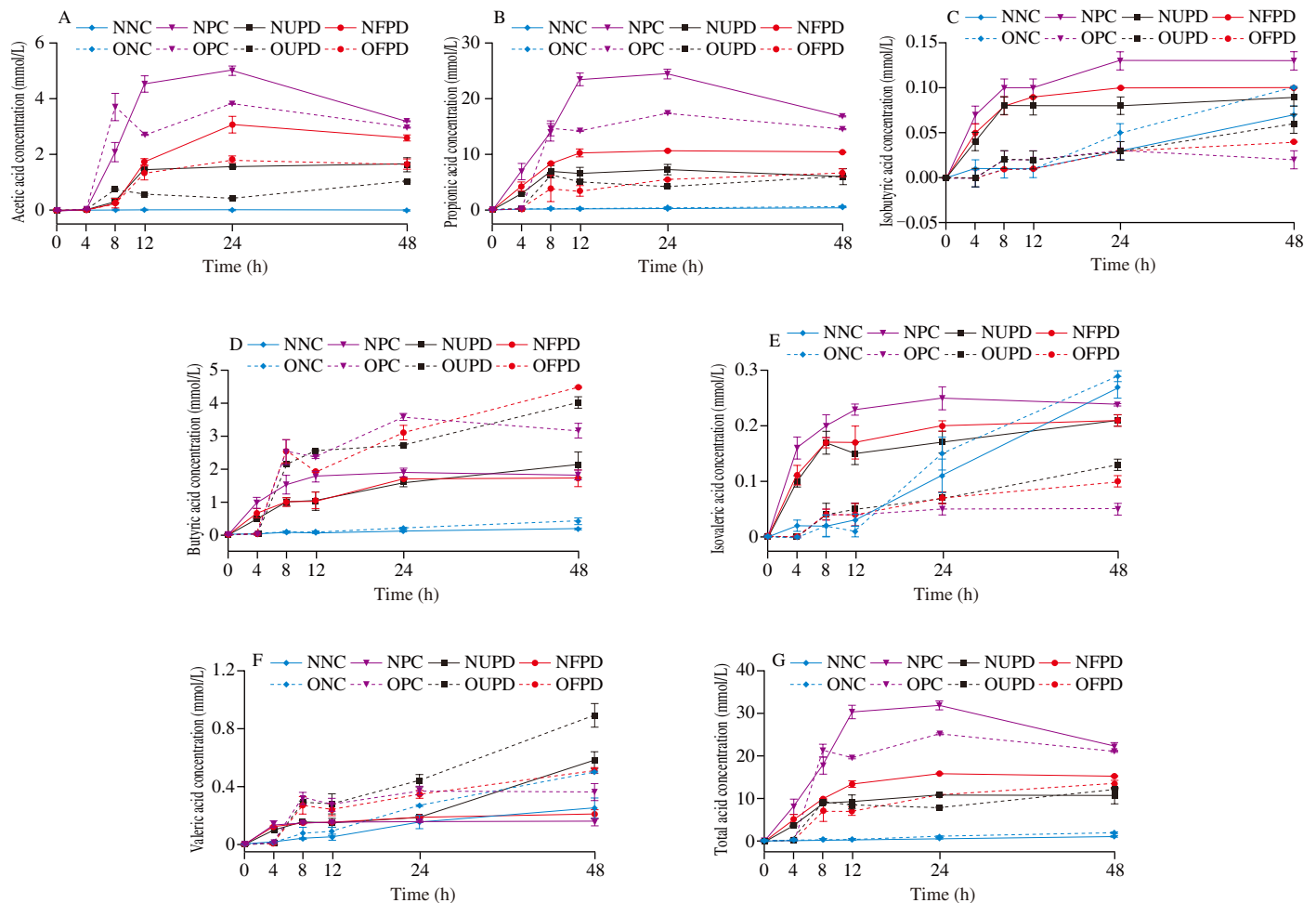


Fig. 3 The content change of (A) acetic acid, (B) propionic acid, (C) isobutyric acid, (D) butyric acid, (E) isovaleric acid, (F) valerate acid, and (G) total acid during simulation of human colon fecal fermentation *in vitro*. (H) The SCFA composition in each treatment group at different fermentation times. (I) The PCA chart of SCFAs. (J) The OPLS-DA chart of SCFAs. The change in (K) LPS, (L) growth hormone, and (M) H_2S content during simulation of human colon fecal fermentation *in vitro*. ** $P < 0.01$, *** $P < 0.001$.

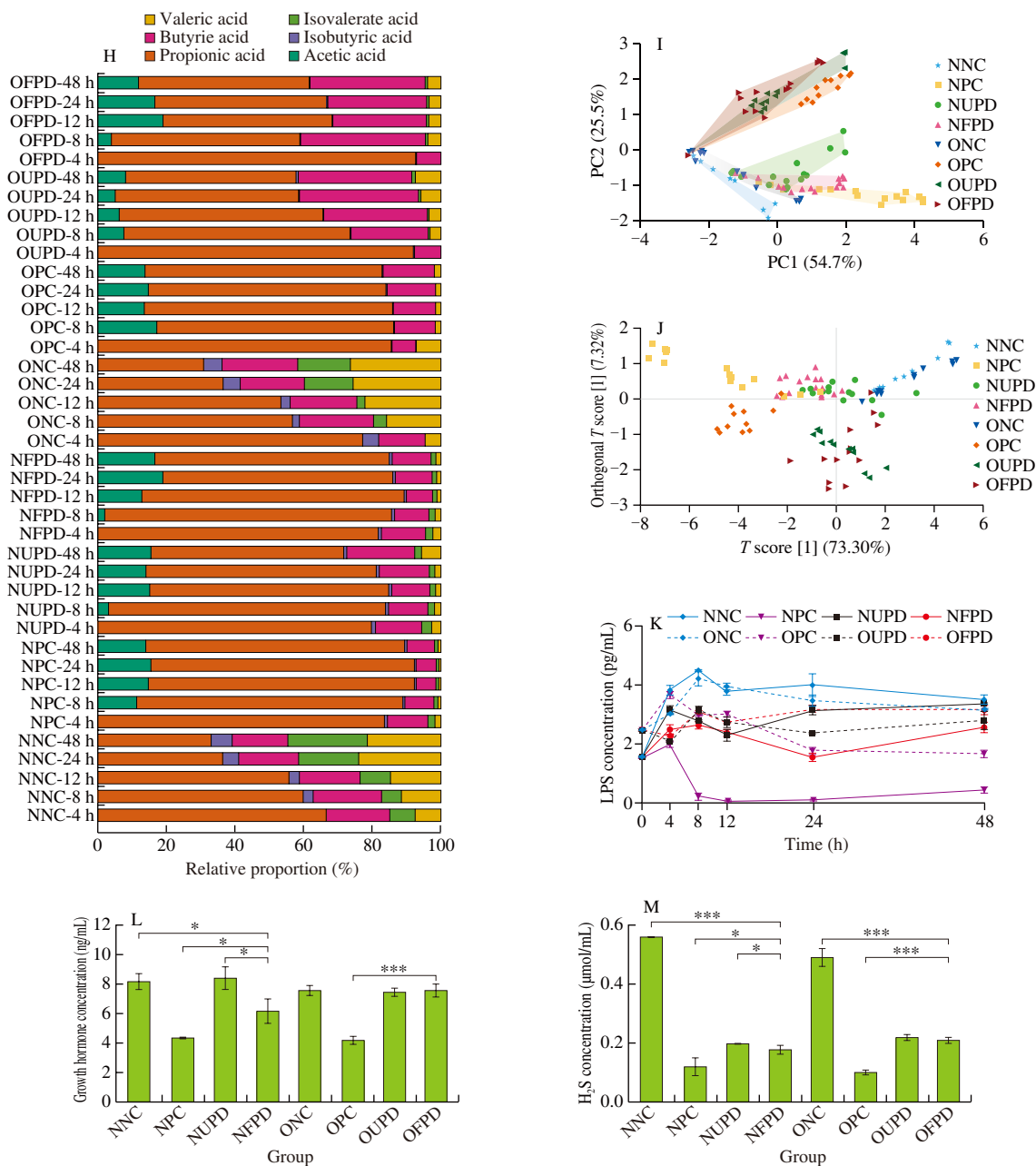


Fig. 3 (Continued)

In addition, H₂S, as an intestinal gas produced through food digestion, its high level increases intestinal pressure but also damages the oxidation of butyrate and other SCFAs; it may lead to nutrient deficiency^[48]. The study results revealed that BHP addition significantly reduced H₂S production at 24 h, and the inhibitory effect of NFPD was significantly higher than that of NUPD. The inhibitory effect of OFPD and OUPD exhibited no significant difference (Fig. 3M).

3.5 Effects of BHP on the SCFA synthesis pathway

To further reveal the action mechanism of BHP affecting microbial metabolism that leads to SCFA production, based on the KEGG database, the Tax4Fun function was used to predict key enzyme activities in SCFA metabolism in different treatment groups.

Accordingly, the potential SCFA synthesis pathway was constructed, so as to further expound the SCFA synthesis mechanism at the enzymological level. On comparing the key enzyme activities involved in acetic acid (76, map00620), propionic acid (52, map00640), and butyric acid (61, map00650) synthesis by using the KEGG database, 16 (acetic acid), 21 (propionic acid), and 15 (butyric acid) key enzyme activities exhibiting significant differences among groups were detected. Furthermore, the potential SCFA synthesis pathways were mapped (Figs. 4 and S5-6).

Acetic acid is synthesized primarily through the acetyl-CoA pathway (Fig. S5), starting with phosphoenolpyruvate, which is a product of the glycolysis pathway^[11,25]. In this study, FP promoted the production of pyruvate, a key substance in acetic acid production, by promoting phosphoenolpyruvate synthase activity in group N. Pyruvate then promoted acetic acid production by promoting

acetyl-CoA production. The enzymes associated with this process were mostly pyruvate dehydrogenase, pyruvate synthase, acetate kinase, and so on. Moreover, pyruvate first directly promoted pyruvate dehydrogenase or pyruvate oxidase activity and then promoted acetic acid generation. Furthermore, phosphoenolpyruvate promoted the activities of phosphoenolpyruvate carboxykinase, pyruvate carboxylase, malate dehydrogenase, and oxaloacetic acid decarboxylase and promoted pyruvate production, thereby promoting acetic acid production, which was similar to the FP-regulated acetic acid generation pathway in group O.

BHP intervention can significantly affect the content of propionic acid. Propionic acid is mainly generated through 3 pathways, namely the succinate pathway, acrylate pathway, and propanediol pathway (Fig. 4)^[11,25]. The first step in the succinate pathway begins with succinyl-CoA produced during the citrate cycle. FP promoted propionic acid production by promoting propanoyl-CoA production in group N, which primarily involved enzymes such as succinate-CoA ligase, propionyl-CoA carboxylase, propionate CoA-transferase, and acetate-CoA ligase. This was similar to the FP-regulated acetic acid generation pathway in group O. Second, the acrylate pathway that is involved in propionic acid generation starts from acetyl-CoA produced through pyruvate metabolism^[11]. The 2-oxobutanoate produced by the amino acid metabolic pathway in group N promoted propanoyl-CoA formation, followed by propionic acid formation. Third, the propanediol pathway that generates propionic acid starts with glycerone-P from glycolysis. FP promoted propanol production by promoting the activities of methylglyoxal synthase, lactaldehyde reductase, and propanediol dehydratase in group N, thereby promoting propionic acid production. The FP-promoted propanediol pathway in group O and N was similar.

Fig. S6 presents the butyric acid synthesis pathway, which starts from *L*-glutamate and 4-aminobutanoate produced through amino acid metabolism^[11,25]. FP promoted the activities of glutamate decarboxylase, *D*-malate dehydrogenase, pyruvate synthase, acetoacetyl-CoA reductase, butyrate kinase, and so on, thereby promoting butyric acid generation in group N. Unlike in group N, FP mainly started from maleate by promoting the activities of *D*-malate dehydrogenase, acetate CoA-transferase, and so on, thereby inducing butyric acid production in group O.

3.6 Correlation analysis between polyphenol composition and microbial metabolites

To determine the effect of BHP composition on microbial metabolites, the correlation between the significantly different polyphenols in UP and FP and acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, LPS, GH, and H₂S was explored (Fig. 5). Fig. 5A presents the correlation results of N. The microbial metabolites were mainly divided into 3 categories, namely acetic acid, propionic acid, isobutyric acid, and isovaleric acid; valeric acid and butyric acid; and LPS, GH, and H₂S. SCFAs exhibited a significant positive correlation with most of the significantly different polyphenols, whereas LPS, GH, and H₂S exhibited significant negative correlations. Rutin, kaempferol-3-*O*-rutinoside, diosmin in flavonoids, epicatechin, cyanidin 3-*O*-rutinoside in flavones, and octopamine in phenols exhibited a significant positive correlation with the SCFAs. Among them, the RP of rutin, epicatechin, and octopamine significantly increased in FP, and rutin (34.29%) and octopamine (13.84%) were the main FP components.

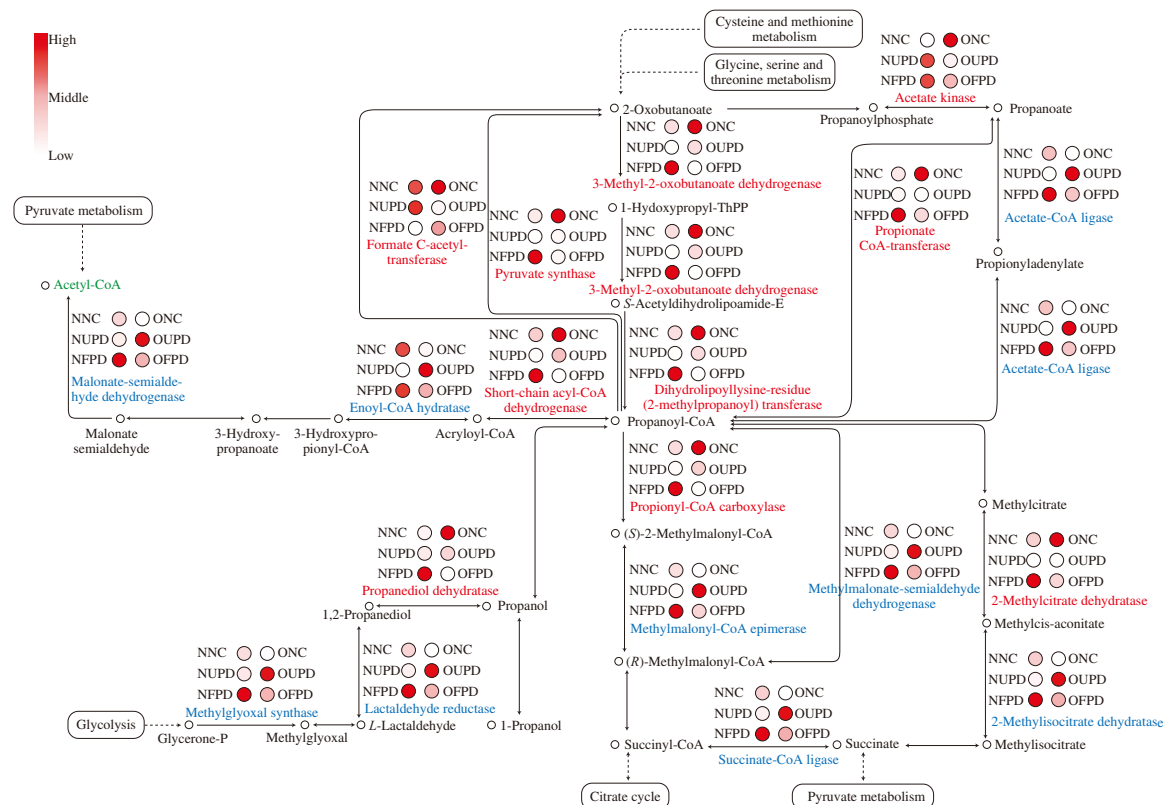


Fig. 4 The potential propionic acid synthesis pathway was mapped according to the effect of BHP on the key enzyme activity during propionic acid metabolism.

Moreover, 9 differential polyphenols in UP and FP, namely rutin, octopamine, epicatechin, cianidanol, diosmin, cyanidin 3-*O*-rutinoside, tricetin, quercetin, and dihydromyricetin, exhibited significant positive correlations with acetic acid, propionic acid, and butyric acid, and negative correlations with LPS (Figs. 5A and B). Except for diosmin and cyanidin 3-*O*-rutinoside, the content of the remaining 7 differential polyphenols significantly increased in FP.

The correlation results of group O are presented in Fig. 5C. The microbial metabolites were mainly clustered into 2 categories: acetic acid, propionic acid, butyric acid, and valeric acid; and isobutyric acid, isovaleric acid, LPS, GH, and H₂S. Rutin and octopamine exhibited significant positive correlations with acetic acid, propionic acid, butyric acid, and valeric acid. Furthermore, 7 differential polyphenols in UP and FP, namely rutin, octopamine, epicatechin, cianidanol, tricetin, quercetin, and dihydromyricetin, exhibited significant positive correlations with acetic acid, propionic acid,

and butyric acid, but a negative correlation with LPS. The content of all of these 7 differential polyphenols significantly increased in FP (Figs. 5C and D).

3.7 Key glycolipid metabolism-related factors

The aforementioned studies confirmed that microbial fermentation can change the BHP composition and further affect microbial metabolite generation. However, whether this change can ultimately intervene in obesity needs to be further explored. Obesity is mainly accompanied by glycolipid metabolism disorder and insulin resistance^[1,57]. Therefore, this study selected key glycolipid metabolism-related cells to explore the mechanism underlying BHP intervention in obesity. First, the optimal polyphenol concentration was determined through the CCK8 assay. The survival rates of small intestinal endocrine cells STC-1 (Fig. S7A), RIN-m5f cells (Fig. S7B),

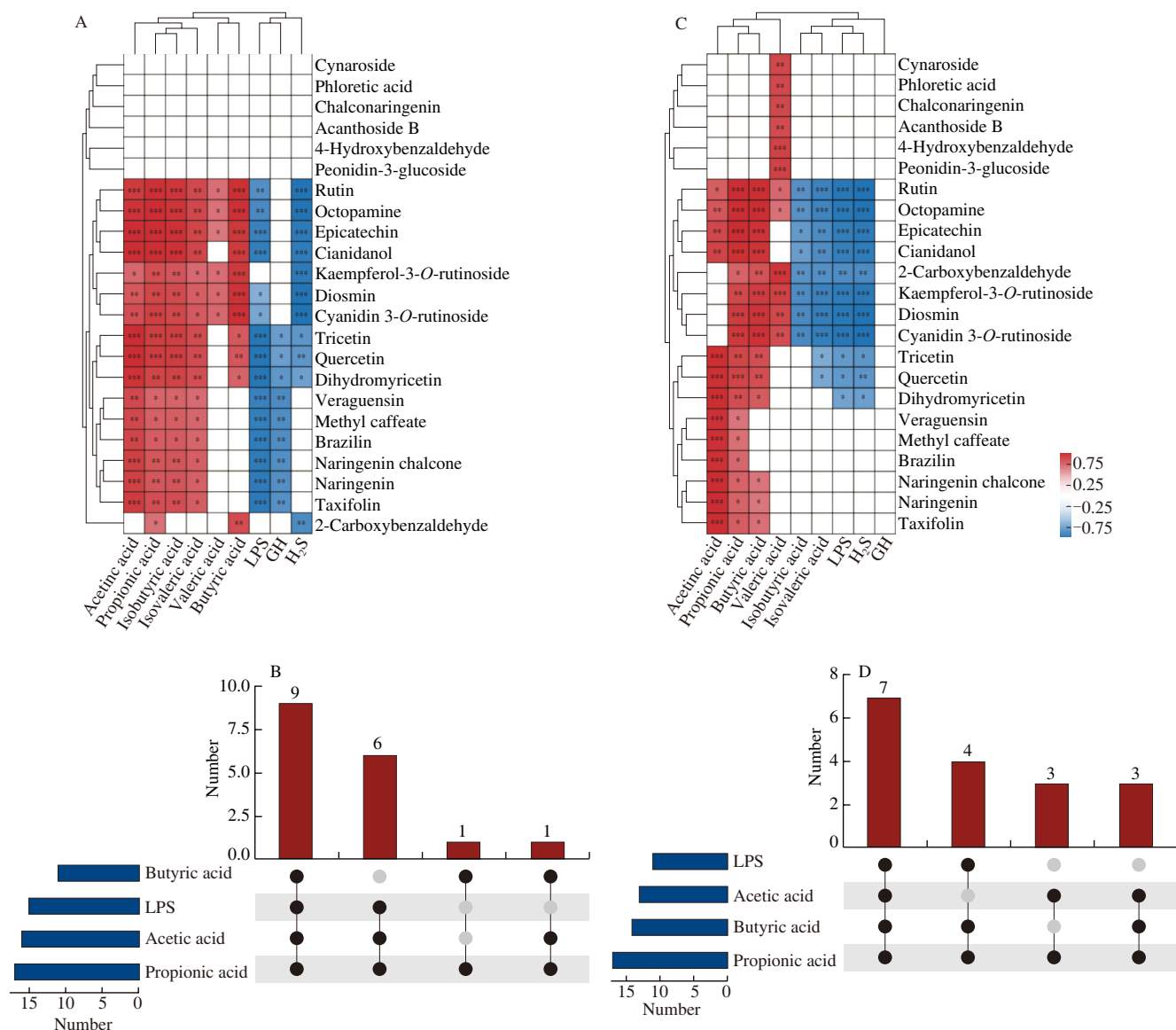


Fig. 5 (A) A heatmap depicting the correlation of polyphenols with microbial metabolites, which was significantly different between UP and FP in the normal fecal group (N). (B) An UpSet map of acetic acid, propionic acid, butyric acid, and LPS-related polyphenols in group N. (C) A heatmap of the correlation between polyphenols and microbial metabolites, which was significantly different between UP and FP in the obese fecal group (O). (D) An UpSet map of acetic acid, propionic acid, butyric acid, and LPS-related polyphenols in group O (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

and RAW264.7 cells (Fig. S7C) were relatively higher ($> 80\%$) at $125 \mu\text{g/mL}$ polyphenol. Thus, the $125 \mu\text{g/mL}$ BHP concentration was selected for subsequent experiments.

The fecal fermentation supernatant of UP and FP *in vitro* and untreated UP and FP were used here to stimulate STC-1 cells. The cell supernatants were collected at 24 and 48 h, and the GLP-1, PYY, and CCK content in the supernatant were determined to explore the BHP intervention effect on beneficial hormones in the gastrointestinal tract. Fig. 6A presents changes in the GLP-1 content after BHP intervention. The GLP-1 content in the experimental group was relatively lower than that in the NNC and ONC groups after 24 h of BHP intervention. However, the GLP-1 content in the NFPD group ($355.06 \pm 2.56 \text{ pg/mL}$) was 19.88% higher than that in the NUPD group. In addition, as the BHP intervention time extended, the GLP-1 content increased in all groups. After 48 h of BHP intervention, compared with NNC ($381.45 \pm 1.86 \text{ pg/mL}$), UP ($393.67 \pm 1.39 \text{ pg/mL}$), and FP ($426.27 \pm 2.58 \text{ pg/mL}$), the GLP-1 content significantly increased in the NUPD ($550.76 \pm 1.84 \text{ pg/mL}$) and NFPD ($431.94 \pm 1.35 \text{ pg/mL}$) groups. This indicated that BHP was more conducive to promoting GLP-1 secretion by STC-1 cells after *in vitro* fecal fermentation. In addition, compared with the ONC group, the GLP-1 content in the OFPD group ($393.58 \pm 1.13 \text{ pg/mL}$) significantly increased by 18.32%, and the promoting effect of OFPD was significantly stronger than that of OUPD. This suggested that FP had a stronger promoting effect on GLP-1 secretion by STC-1 cells than UP. Overall, after *in vitro* fermentation, UP (normal feces) and FP (normal feces and obese feces) can significantly promote GLP-1 secretion by stimulating STC-1 cells for 48 h.

Fig. 6B presents the changes in PYY content after the BHP intervention. After 24-h BHP intervention, OFPD significantly promoted PYY secretion compared with ONC, and the promotion effect of OFPD was significantly stronger than that of OUPD. After 48-h BHP intervention, compared with NNC, the PYY content in NUPD ($3.48 \pm 0.01 \text{ ng/mL}$) and NFPD ($4.80 \pm 0.01 \text{ ng/mL}$) significantly increased by 20% and 65.52%, respectively, and the PYY content in NFPD was significantly higher than that in FP ($3.84 \pm 0.01 \text{ ng/mL}$). This indicated that fecal fermentation could significantly increase the BHP intervention effect on PYY, and the effect of FP was stronger than that of UP. Furthermore, the PYY content in OFPD ($3.10 \pm 0.01 \text{ ng/mL}$) significantly increased by 7.27% compared with ONC. Overall, BHP had a similar effect on PYY secretion by STC-1 cells to that of GLP-1 secretion. UP (normal feces) and FP (normal feces and obese feces) significantly promoted PYY secretion by stimulating STC-1 cells for 48 h after *in vitro* fermentation. Moreover, PYY secretion also increased with an increase in BHP duration, especially in group N.

Changes in CCK content after BHP intervention is presented in Fig. 6C. After 24-h BHP intervention, the CCK content in NFPD ($153.01 \pm 0.37 \text{ pg/mL}$) increased by 5.63%, 11.91%, and 9.65% compared with NNC, NUPD, and FP, respectively. These results indicated that FP had improved the promoting effect on STC-1 cells after *in vitro* fecal fermentation (normal feces). In addition, after 48-h BHP intervention, the CCK content significantly decreased in NUPD and NFPD compared with NNC, but CCK content significantly increased in OFPD compared with ONC. In summary, different from GLP-1 and PYY content, the content of STC-1 cell-secreted CCK was lower than that in the control group when the polyphenol intervention

time was extended. This indicated that polyphenol intervention can promote CCK secretion by STC-1 cells at the initial stage.

INS has a crucial role in maintaining blood glucose stability^[56]. Therefore, UP and FP after *in vitro* fecal fermentation, as well as untreated UP and FP were used here to stimulate RIN-m5f cells. The cell supernatant was collected at 24 and 48 h, and the INS content was determined. Fig. 6D presents the changes in the INS content after the BHP intervention. After 24-h BHP intervention, compared with NNC ($0.58 \pm 0.03 \text{ mU/L}$) and ONC ($0.48 \pm 0.12 \text{ mU/L}$), the INS content in NUPD, NFPD, OUPD, and OFPD increased by 2.19, 5.43, 2.38, and 3.04 times, respectively. The INS content was significantly higher in NFPD and OFPD than in NUPD and OUPD. Moreover, the INS content was higher in NFPD ($3.15 \pm 0.05 \text{ mU/L}$) than in OFPD ($1.46 \pm 0.05 \text{ mU/L}$). After 48 h of BHP intervention, except for that in OUPD, BHP intervention significantly reduced the INS content in all groups compared with the NNC and ONC groups. Overall, BHP stimulation of RIN-m5f cells for 24 h after *in vitro* fermentation (normal feces and obese feces) significantly promoted INS secretion by the cells, and the effect of FP was stronger than that of UP. Additionally, UP significantly promoted INS secretion by stimulating RIN-m5f cells for 48 h after *in vitro* fecal fermentation (obese feces).

The supernatant of UP and FP after *in vitro* fecal fermentation and untreated UP and FP stimulated RAW264.7 cells. The cell supernatants at 24 and 48 h were collected to determine IL-6 and TNF- α content to explore whether BHP intervention inhibited inflammation. Fig. 6E presents changes in the IL-6 content after BHP intervention. After 24-h BHP intervention, compared with NNC ($8.71 \pm 0.12 \text{ pg/mL}$) and NUPD ($9.52 \pm 0.31 \text{ pg/mL}$), the IL-6 content in NFPD ($7.94 \pm 0.70 \text{ pg/mL}$) decreased by 8.84% and 16.60%, respectively. The IL-6 content was significantly lower in NFPD than in NUPD. Compared with ONC ($14.44 \pm 0.12 \text{ pg/mL}$), OUPD ($11.26 \pm 0.08 \text{ pg/mL}$), and FP ($7.24 \pm 0.51 \text{ pg/mL}$), OFPD ($1.07 \pm 0.18 \text{ pg/mL}$) significantly reduced the IL-6 content. Additionally, the IL-6 content was significantly lower in OFPD than in NFPD. After 48-h BHP intervention, NFPD significantly reduced the IL-6 content compared with NNC and NUPD. OFPD also significantly reduced the IL-6 content compared with ONC, OUPD, and FP. Overall, RIN-m5f cells stimulated by BHP through *in vitro* fecal fermentation significantly inhibited IL-6 secretion. The effect of FP was stronger than that of UP. Fig. 6F present changes in the TNF- α content after BHP intervention. After BHP intervention for 24 and 48 h, compared with NUPD (61.55 ± 1.58), ($131.64 \pm 2.52 \text{ pg/mL}$) and OUPD (65.42 ± 1.00), ($58.06 \pm 0.68 \text{ pg/mL}$), NFPD (38.61 ± 1.14), ($61.24 \pm 1.89 \text{ pg/mL}$) and OFPD (39.48 ± 0.84), ($47.79 \pm 1.21 \text{ pg/mL}$) significantly reduced the TNF- α content. Furthermore, UP had no potential to inhibit TNF- α secretion, and the inhibitory effect of FP on TNF- α decreased as the intervention time was extended.

In summary, compared with the blank control, the supernatant of BHP after *in vitro* fecal fermentation significantly promoted GLP-1, PYY, CCK, and INS secretion and inhibited IL-6 secretion, and the effect of FP was better than that of UP.

3.8 Correlation analysis between SCFAs and key glycolipid metabolism-related factors

Along with cell experiment results, significant differences were observed in the intervention effects of FP and UP on the key

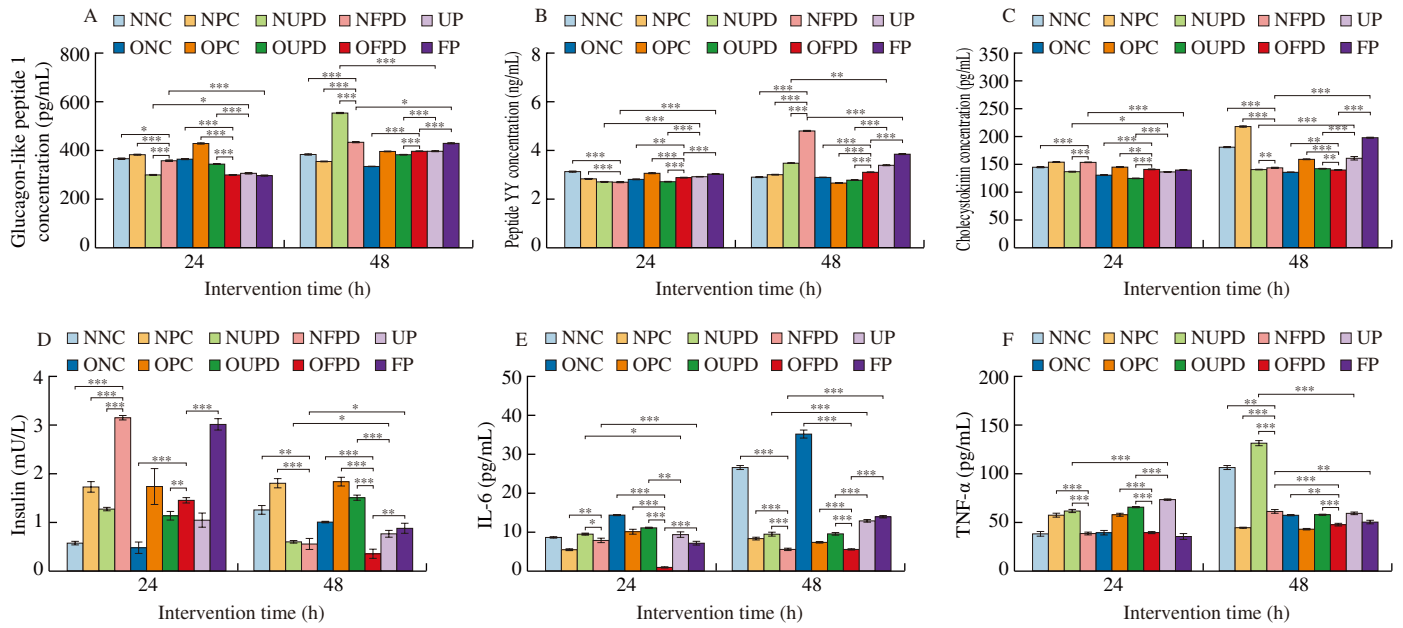


Fig. 6 *In vitro* fecal fermentation supernatant of UP-, FP-, untreated UP-, and untreated FP-stimulated STC-1 cells. The cell supernatant was collected at 24 and 48 h to determine the (A) GLP-1, (B) PYY, and (C) CCK content. Islet β tumor cells (RIN-m5f) were stimulated to determine the (D) insulin content. Mononuclear macrophage cells (RAW264.7) were stimulated to determine the (E) IL-6 and (F) TNF- α content. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

glycolipid metabolism-related factors. To further clarify whether such differences were observed due to microbial metabolites, a correlation analysis of these key factors and microbial metabolites (SCFAs and LPS) was conducted. The key microbial metabolites significantly influencing the key glycolipid metabolism-related factors were screened out (Fig. 7). The results revealed that acetic acid and propionic acid were significantly positively correlated with GLP-1, CCK, and INS, but negatively correlated with IL-6 ($P < 0.05$). Butyric acid was significantly negatively correlated with IL-6 ($P < 0.05$), and LPS was significantly negatively correlated with CCK and INS but positively correlated with IL-6 ($P < 0.05$). These results suggest that acetic acid and propionic acid mainly promote beneficial hormones in the gastrointestinal tract, butyric acid mainly inhibits

inflammatory cytokines, and LPS inhibits beneficial hormones in the gastrointestinal tract and promotes inhibitory inflammatory cytokines. SCFAs produced through microbial metabolism thus played a positive regulatory role in the body's glycolipid metabolism, whereas LPS played a negative regulatory role.

4. Discussion

This study aimed to screen for strains that can utilize BH as the sole carbon source for growth and to increase the content and activity of BHP formed during fermentation. This study screened the most suitable fermentation strain *L. rhamnosus* 6224 and *S. cerevisiae* W5 for BH fermentation from among 17 LAB and

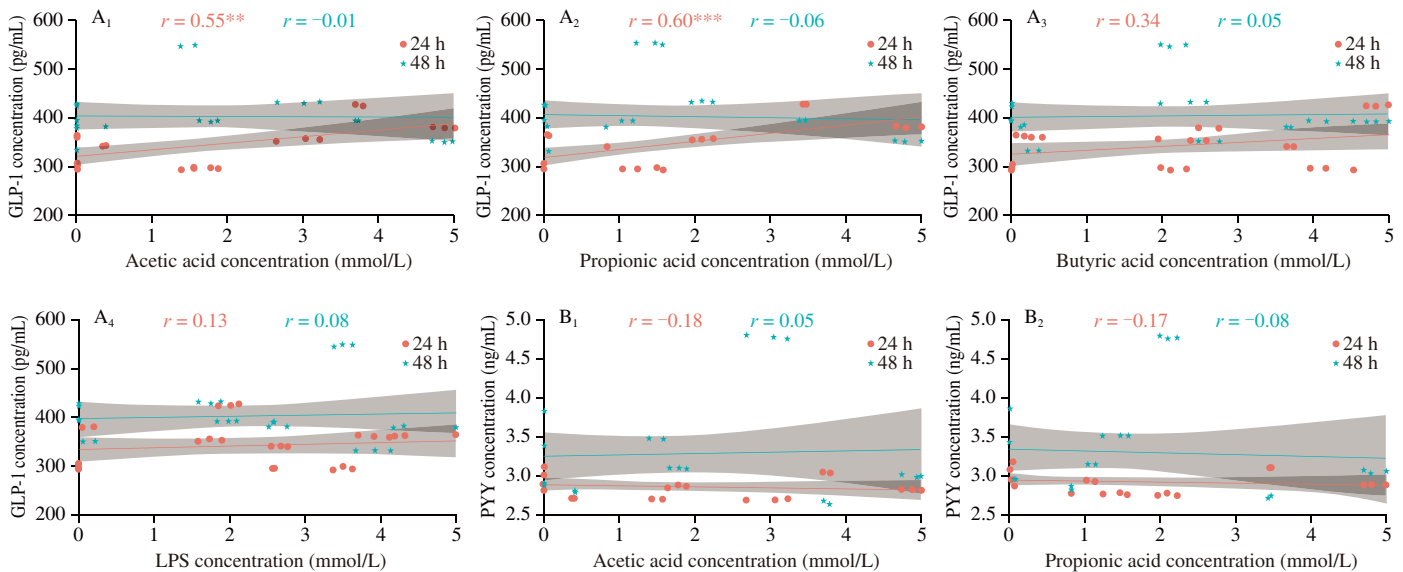


Fig. 7 Linear regression analysis of microbial metabolism-mediated production of SCFAs and LPS and the secretion of (A) GLP-1, (B) PYY, (C) CCK, (D) INS, (E) IL-6, and (F) TNF- α by glycolipid metabolism-related cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

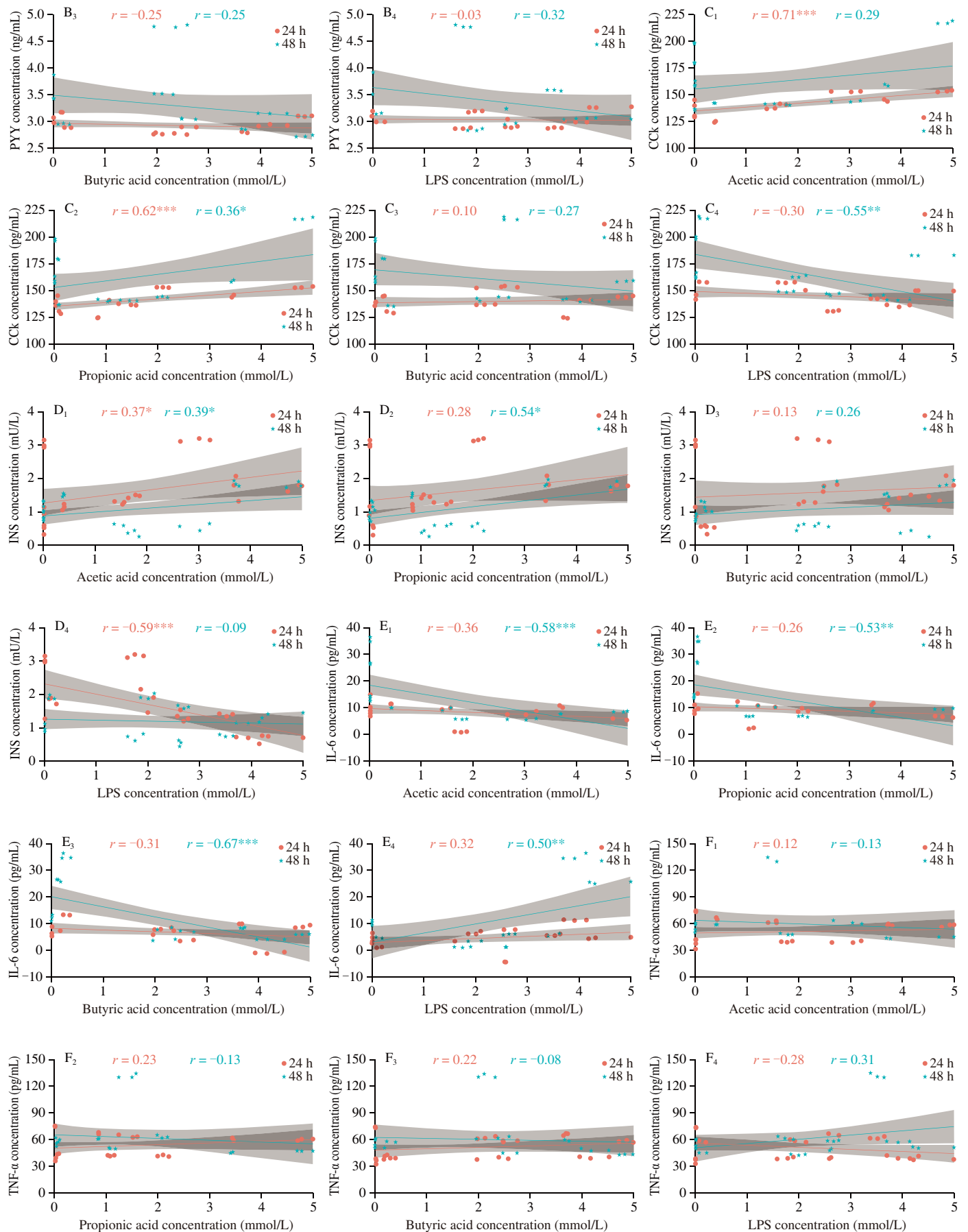


Fig. 7 (Continued)

3 yeast strains by considering a live bacterial count, polyphenol content, pH value, and DPPH free radical scavenging ability as indicators. The total free polyphenol content of *L. rhamnosus* 6224 after fermentation was significantly higher than that of *L. plantarum* TR-71 ((202.65 ± 0.35) mg/100 mL). This may be because the fermentation strain and substrate influence the polyphenol content^[28]. Meanwhile, *S. cerevisiae* W5 fermentation can maintain a high pH of BPH, preventing the excessive acid-mediated inhibition of strain growth, which is consistent with the results of de Lourdes Chaves Macêdo et al.^[31]. They found no significant change in the pH of the soursop pulp before and after yeast fermentation. However, during *L. rhamnosus* 6224 fermentation, the pH of the BH pulp decreased, which is consistent with the results of de la Fuente et al.^[28]. The mixed fermentation method of LAB and yeast is thus inferred to compensate for the shortcomings of single bacterial fermentation, maintain the pH value of the fermentation system, and facilitate obtaining more highly active polyphenols.

The health-promoting effect of polyphenols is mainly attributable to their strong antioxidant potential and radical scavenging ability, thereby delaying lipid and protein oxidation^[34]. Therefore, this study investigated the BHP antioxidant ability when exploring BHP intervention in glycolipid metabolism. Moreover, because of the different action mechanisms of radicals, the results of different antioxidant experiments may vary^[22]. Therefore, in this study, 5 antioxidant methods were selected to evaluate the effects of fermentation of different strains on the BHP antioxidant ability, thereby avoiding the one-sided results of a single method. The study results revealed that after fermentation, the BHP antioxidant activity increased in a concentration-dependent manner; similar results were reported by Zhang et al.^[37] and Zhou et al.^[14]. These authors studied the fermentation of *Aspergillus niger* and Pu'er tea residue for beverages. Meanwhile, the BHP content in the *L. rhamnosus* 6224 and *S. cerevisiae* W5 mixed fermentation was significantly higher than that in the unfermented group. This is consistent with the results of blueberry fermentation by Li et al.^[27] and Zhang et al.^[38]. These authors showed that the total phenol content significantly increased after fermentation.

When α -amylase and α -glucosidase activities are inhibited, circulating glucose levels are maintained by reducing blood glucose absorption^[7,54]. Existing enzyme inhibitors such as acarbose, miglitol, and vogliose are used to inhibit the activity of the aforementioned enzymes. However, many side effects, such as gastrointestinal disorders, exist. Therefore, extracting substances from natural plants that inhibit α -amylase and α -glucosidase activities is of considerable significance for obesity prevention and treatment^[58]. In this study, the evaluation results of the *in vitro* hypoglycemic effect of polyphenols revealed that BHP had the strongest inhibitory effect on α -amylase, and polyphenols exerted a dose-dependent inhibitory effect on α -amylase and α -glucosidase, which is consistent with the results of Deng et al.^[54]. These researchers found that the hullless barley polyphenol extract had certain dose-dependent inhibitory effects on α -amylase and α -glucosidase. In summary, combined with the evaluation results of polyphenol content, antioxidant activities, and glycolipid metabolism *in vitro*, *L. rhamnosus* 6224 and *S. cerevisiae* W5 mixed fermentation of BH resulted in the best polyphenol content and activity. The mixed fermentation method was therefore used in subsequent experiments.

In response surface testing, a statistical method, multiple variables, and their interactions are solved through a reasonable experimental design; it is a typical optimization technique^[59-61]. At present, numerous studies have optimized the process of polyphenol extraction from various plants by using response surface methodology. Li et al.^[60] used this methodology to optimize the total anthocyanin and total phenol content, and antioxidant activity of the aqueous BH extract. Pinto et al.^[61] used the response surface method to investigate the subcritical water extraction process of antioxidants and polyphenols from chestnut shells. Clodoveo et al.^[59] optimized ultrasonic-assisted polyphenol extraction from the carob pulp by using the response surface method. Therefore, we used the PB experiment to screen out the significant and positive effects of the berry ratio, strain rate, and inoculum dose on the BHP content. To further improve the polyphenol content, CCD experiments were conducted. The results revealed that when the berry ratio was 1:2.2, the temperature was 34 °C, the shaking speed was 160 r/min, *S. cerevisiae* W5 to *L. rhamnosus* 6224 ratio was 1:2.8, the inoculum dose was 5%, and time was 42 h, the BHP content reached $(1\,878.08 \pm 14.53)$ mg/100 g, which was 2.63 times of the initial BHP content.

Polyphenols can be potentially used as functional foods, nutritional drugs, or pharmaceutical ingredients^[15,17]. Meanwhile, because people are on a continuous quest for a healthy quality of life, research has focused on the relationship between dietary health and bioactive ingredients in plants^[15,17]. Furthermore, because BH has the potential to be used as a functional food and nutritional drug or pharmaceutical ingredient, compounds in BH that offer health benefits must be identified^[19]. Flavones are the main phenolic compounds in fruits. They play a crucial role in color, taste, enzyme and microbial synthesis, and anti-inflammation and lipid peroxidation, and have α -amylase and α -glucosidase inhibitory activities *in vitro*^[17,58]. The RP of flavones in FP increased by 49.88% compared with UP, which is consistent with the results of Li et al.^[27]. They noted that the content of flavones (rutin) significantly increased after blueberry fermentation. Therefore, FP may have a stronger intervention ability for obesity, which will be verified in subsequent tests. Additionally, the RP of carbohydrates in FP decreased by 20.21% compared with UP. This indicated that microbial fermentation could use carbohydrates (glucose and fructose) in BH, which is consistent with the results of de la Fuente et al.^[28] and de Lourdes Chaves Macêdo et al.^[31]. Flavonoids are mainly composed of anthocyanins. Anthocyanins give fruits a colorful appearance and have anti-inflammatory, anticancer, and antiobesity effects^[17]. In the present study, the RP of flavonoids in FP was reduced compared with those in UP, which is consistent with the results of blueberry fermentation by Li et al.^[27] and Oh et al.^[26]. The content of flavonoids (anthocyanins) after fermentation is significantly reduced, which might be because some flavonoids are destroyed by microbial fermentation. Meanwhile, the color of the BH fermentation broth was lighter than that of the unfermented. This may be caused by microbial degradation and the adsorption of some anthocyanins in flavonoids^[27]. In addition, the results of the differential metabolite analysis revealed 23 significantly different phenolic metabolites between UP and FP. Among them, mixed bacterial fermentation significantly increased the RP of 13 metabolites, whereas decreased the RP of 10 metabolites. Although the RP of some metabolites such as kaempferol-3-*O*-rutinoside decreased, the total free polyphenol content increased. This indicated

that enzymes produced through microbial metabolism can help transform polyphenols^[31].

In addition, the RP of rutin in differential metabolites was relatively high. The RP in FP and UP was 43.71% and 32.97%, respectively. Rutin mainly existed in the free part of flavones^[50]. Rutin was significantly higher in FP than in UP, indicating that microbial fermentation could transform some of the bound rutin into the free state. The rutin content in blueberry juice increased by 136% after LAB fermentation for 48 h, and the *in vitro* antioxidant ability increased by at least 34.0%^[27]. The antioxidant activities of mulberry juice after LAB fermentation were related to the post-fermentation phenolic profile^[21]. Therefore, the increase in rutin content enhances its biological activity. In summary, the BHP composition changed significantly after *L. rhamnosus* 6224 and *S. cerevisiae* W5 mixed fermentation.

As the main research means for studying the relationship between polyphenols and gut microbiota metabolites, *in vitro* simulated gastrointestinal digestion and colonic fermentation models offer the advantages of low cost, short time, and strong controllability, and have recently been used extensively. Ashley et al.^[49] investigated the intervention effect of the sorghum bran polyphenol extract on obesity by simulation human colon fermentation *in vitro*. Burgos-Edwards et al.^[47] studied the effect of Chilean currants polyphenol on gut microbiota metabolites by simulating gastrointestinal digestion and human colon fermentation *in vitro*. Therefore, the present study explored the effects of BHP on gut microbiota metabolites by simulating gastrointestinal digestion and colon fermentation *in vitro*.

Metabolites produced through microbial metabolism during *in vitro* fecal fermentation is known to affect the pH value of the system. Reducing colon pH to an appropriate extent can effectively inhibit pathogen proliferation, facilitate probiotic colonization in the intestinal cavity, and have a beneficial effect on human intestinal health^[46-47]. The pH value, as an indirect indicator of the fermentation performed by intestinal microorganisms, can reflect whether the exogenous substances added have a regulatory effect on gut microbiota^[47]. Therefore, to investigate whether BHP can affect gut microbiota behavior, the pH value of the fermentation system was first detected. The results revealed that FP significantly reduced the pH value of the system. Burgos-Edwards et al.^[47] also reached a similar conclusion, which was attributable to the Chilean currants polyphenol fermented by the gut microbiota to produce large amounts of SCFAs and reduce environmental pH. The pH value of FP was speculated to be lower because more SCFAs were produced.

SCFAs are organic fatty acids with 1–6 carbon atoms and mainly include acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and caproic acid^[18]. Of these SCFAs, acetic acid, propionic acid, and butyric acid are the most abundant SCFAs in the human gut, accounting for approximately 86% of the total SCFAs^[1]. SCFAs can directly affect carbohydrate metabolism and lipid utilization. Moreover, SCFAs are a crucial energy source for intestinal epithelial cells, play a vital role in maintaining intestinal epithelial function, and can alleviate obesity- and diabetes-related symptoms^[25,62]. The study results revealed that whether in groups O or N, the promoting effect of FP on microbial metabolism to produce acetic acid is significantly higher than that of UP. Therefore, FP can be inferred to be more conducive to promoting microbial metabolism to acetic acid production. Wang et al.^[12] obtained similar results in the intervention of obesity with *Morinda citrifolia* L. polyphenols.

Propionic acid is absorbed by colon cells and metabolized in the liver through gluconeogenesis. Meanwhile, propionic acid can inhibit cholesterol and fatty acid synthesis^[62]. In this study, the promoting effect of FP on microbial metabolism to produce propionic acid was significantly higher than that of UP. Therefore, FP can be inferred to be more conducive to promoting microbial metabolism to propionic acid production. Moreover, after BHP intervention, the propionic acid content was significantly higher in group N than in group O. However, Ashley et al.^[49] exhibited that the propionic acid content was higher in the normal weight group than in the overweight/obese group, but not significantly. This was possible because the gut microbiota of normal and obese participants is different, which may lead to differences in the composition and content of propionic acid-producing microorganisms. Once BHP intervention was administered, the regulation ability of the gut microbiota was different, and the propionic acid-producing bacteria in group N were more likely to utilize BHP. Butyric acid is involved in multiple metabolic pathways in the body, including gluconeogenesis and ketosome formation, and is produced by most intestinal bacteria^[62]. In this study, the BHP intervention revealed that the butyric acid content was significantly higher in group O than in group N. Therefore, polyphenols can be inferred to have a relatively greater impact on the composition of butyric acid-producing microorganisms in group O, and the intervention ability of FP was stronger. No significant difference in total acid content was observed between NUPD and OUPD ($P > 0.05$), whereas a significant difference was observed between NFPD and OFPD ($P < 0.05$). This indicated that no significant difference existed in the regulatory effect of UP on SCFA production through microbial metabolism in groups N and O. While the regulatory effect of FP on SCFA production through microbial metabolism in group N was significantly stronger than that in group O. However, Ashley et al.^[49] measured the total SCFA content after intervening with the sorghum bran polyphenol extract in normal and obese weight groups. They found no significant difference in the total acid content between the 2 groups, possibly because the composition of different polyphenols is different, and the intervention ability for microbial metabolism to produce SCFAs is different^[47,55].

To further investigate the effect of BHP on SCFA generation, the SCFA composition in different fermentation time points was compared. After BHP intervention was administered, SCFAs in each group were mainly composed of propionic acid, butyric acid, and acetic acid. However, Burgos-Edwards et al.^[47] reported that acetic acid, propionic acid, and butyric acid accounted for 95% of the measured SCFAs, with a ratio of approximately 60:20:20. This may be because the composition of polyphenols from different sources exhibits significant differences, and the composition of SCFAs produced by the gut microbiota through polyphenol metabolism is also different. This proves that polyphenol composition can significantly affect the metabolites of the gut microbiota. Therefore, the relationship between the BHP composition and the gut microbiota metabolites was also explored in this study. LPS, a metabolite of intestinal pathogenic microbes, can exacerbate inflammation and insulin resistance in the body^[9-11]. Therefore, this study evaluated the inhibitory effect of BHP on LPS production. Compared with UP, FP significantly inhibited LPS production in group N, but the effects of both UP and FP on LPS production in group O were not significant. The ability of FP to interfere with gut microbiota metabolism involved in LPS production was better in normal-weight participants than in obese participants.

The most important method of SCFA synthesis is endogenous fermentation by gut bacteria. The gut microbiota decomposes dietary fiber into monosaccharide and oligosaccharide molecules through a series of microbial enzymatic reactions, further generating acetic acid, propionic acid, butyric acid, and other SCFAs. These enzymes decompose polysaccharides as a crucial step in SCFA synthesis^[25,47]. In addition, different bacteria synthesize SCFAs with different efficiencies. The same species may produce different SCFAs under different conditions because enzymes produced by specific bacteria have a preference for a substrate^[25]. SCFA-producing bacteria are associated with various metabolic pathways in which the catalytic efficiency of key enzymes varies. A detailed understanding of the main metabolic pathways, rate-limiting steps, and related key enzymes of SCFA synthesis is of urgent significance for clarifying the metabolic characteristics of SCFAs and reasonably regulating their levels. The study results revealed that UP and FP affect microbial metabolism involved in the SCFA production pathway by affecting different key enzymes.

A comprehensive analysis of the correlation between groups N and O revealed 7 common differential polyphenols in UP and FP. These polyphenols were significantly and positively correlated with acetic acid, propionic acid, and butyric acid, whereas significantly and negatively correlated with LPS. They belong to flavonoids (rutin, cyanidanol, quercetin, dihydromyricetin, flavones (tricetin, epicatechin), and phenols (octopamine). Meanwhile, the content of these 7 common differential polyphenols significantly increased in FP. The RP of rutin (34.29%), octopamine (13.84%), and tricetin (6.53%) were relatively high in FP. Therefore, microbial fermentation was speculated to change the BHP composition, thereby increasing the effect of BHP on microbial metabolites. These 7 differential polyphenols may be the main substances leading to differences in the effects of FP and UP on microbial metabolites.

To better explain the mechanism underlying glycolipid metabolism regulation of BHP, this study selected key cells related to glycolipid metabolism and measured the release of endogenous gut hormones (EGHs), INS, and inflammatory factors after BHP intervention. After a 48-h intervention, FP significantly promoted GLP-1 and PYY secretion compared with UP after *in vitro* fecal fermentation. Polyphenols interact with the gut microbiota. Gut microbiota-produced SCFAs can trigger cell-specific signaling cascades by binding to GPR41 and GPR43 receptors to stimulate the production of the intestinal INS-promoting hormone GLP-1 in L cells, thereby achieving weight loss^[1,7-9,56]. Additionally, SCFAs can increase glucose-stimulated INS secretion through GPR43. Among these SCFAs, propionate can enhance glucose-stimulated INS release and maintain β cell mass by inhibiting apoptosis^[56]. The SCFA-GPR axis thus plays a crucial role in the control of INS secretion and β cell function^[56]. The study results showed that after the 24-h intervention, FP significantly promoted INS secretion after *in vitro* fecal fermentation. Inflammation is the main factor leading to insulin resistance and glycolipid metabolism disorder, while SCFAs produced by the gut microbiota using exogenous dietary substrates activate receptors on the surface of neutrophils, macrophages, and dendritic cells, thereby promoting anti-inflammatory IgA and GLP-1 production^[1]. The study results demonstrated that FP could significantly inhibit IL-6 secretion after *in vitro* fecal fermentation. Wang et al.^[13] revealed that BHP could interfere with the body's fat

absorption ability by regulating inflammatory pathways, which is consistent with our study results. BHP is speculated to intervene in obesity by inhibiting inflammatory pathways.

Combined with previous study results, microbial fermentation of BHP can be speculated to have exerted a better intervention effect on obesity, which may be mainly because microbial BHP fermentation could more effectively promote SCFA production through intestinal microbial metabolism, inhibit LPS formation, and further promote the secretion of EGHs (GLP-1, PYY, CCK) in the gastrointestinal tract, inhibit the production of inflammatory cytokines (IL-6), and increase INS secretion (Fig. 8). However, limitations (closed system, substrate restriction, single-dose treatment) exist in fecal fermentation experiments conducted only *in vitro*, which may cause differences in intervention effects between *in vitro* and *in vivo*. Therefore, before applying the mixed bacterial fermentation of BHP to actual production, *in vivo* experiments must be conducted to further confirm the intervention effect of BHP on obesity.

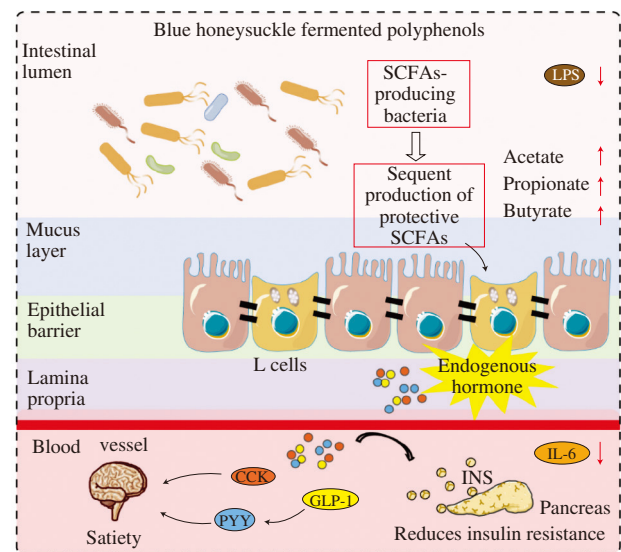


Fig. 8 Mechanism diagram of the intervention of obesity through mixed fermentation of *L. rhamnosus* 6224 and *S. cerevisiae* W5 with BHP.

5. Conclusion

This study mainly simulated human colon fermentation and explored the *in vitro* alleviating effect of BHP on obesity. Regarding novelty, this study is the first to use LAB and yeast mixed fermentation to obtain more free polyphenols from BH, and the fermentation conditions were optimized. Microbial fermentation not only increased the content of free polyphenols in BH but also changed their composition, thereby increasing the biological activity of free polyphenols and promoting gut microbiota metabolism to produce SCFAs. Moreover, cell experiments confirmed that mixed bacteria-fermented BHP after *in vitro* fecal fermentation significantly promoted the secretion of GLP-1, PYY, CCK, and INS by cells, whereas inhibited the secretion of IL-6, which had potential preventive and therapeutic effects on obesity. In summary, *L. rhamnosus* 6224 and *S. cerevisiae* W5 mixed fermentation enhanced the ability of gut microbiota metabolism to produce SCFAs and inhibited LPS generation by increasing the 7 polyphenol content. At the same time, BHP promoted the secretion of beneficial hormones

in the gastrointestinal tract, inhibited the production of inflammatory factors, and ultimately, increased INS secretion, which played a role in the intervention for obesity. The study results provide a theoretical basis for the follow-up *in vivo* study of mice as well as for the use of BHP in obesity prevention and treatment.

Declaration of competing interest

The authors declare no conflict of interest and unanimously approve publication of the manuscript.

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Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2024.9250091>.

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