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Hydroxypropyl-beta-Cyclodextrin embedded resveratrol regulates gut microbiota to prevent NAFLD via activating AMPK signaling pathway

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) has become a globally rising issue that can cause liver-related morbidity and mortality. Recent studies have revealed that a high-fat diet (HFD) highly contributes to the prevalence and progression of NAFLD via impacting gut microbiota and lipid metabolism signal pathways. Resveratrol (RSV), a natural bioactive compound, has exhibited potential for preventing and alleviating NAFLD. However, due to the poor bioavailability of RSV, its strengths and underlying mechanisms for NAFLD therapeutic potential are poorly understood. To address this, we utilized Hydroxypropyl-beta-Cyclodextrin (HBC) to encapsulate RSV to enhance its water solubility and conducted prevention and intervention experiments in HFD-fed mice. The results showed that the HBC has significantly enhanced the water solubility of RSV by 250-fold and the HBC-RSV better prevented and alleviated the liver steatosis, obesity and abnormal lipid metabolism induced by HFD than RSV alone. Meanwhile, combining HFD and HBC-RSV or RSV prevented HFD mice progressing to NAFLD. Besides, further investigation indicated that RSV could resist liver injury and obesity by modulating gut microbiota, raising the levels of short-chain fatty acids (SCFAs) and activating AMP-activated protein kinase (AMPK) signaling pathway. The activated pathway down-regulated sterol receptor element binding protein 1c (SREBP1c) and acetyl-coenzyme A carboxylase (ACC) to decrease lipid synthesis and up-regulated peroxisome proliferators-activated receptorα (PPARα) to promote the fatty acid oxidation, thus preventing NAFLD. Our findings suggested that water solubility-enhanced RSV beneficially modulated gut microbiota, altered gut microbiota-derived SCFAs, and activated lipid metabolism regulatory pathways, providing potential for NAFLD prevention and alleviation.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease characterized by the pathologic lipid deposition in the liver and its incidence is globally rising (Corey & Kaplan, 2014; Huang et al., 2021; Ye et al., 2020). Recent studies have reported that nearly 25% of the world's population was estimated to have NAFLD, and up to 46.9% of patients with NAFLD could progress to the advanced stage with non-alcoholic steatohepatitis (NASH), which increases the risk of outcomes towards cirrhosis, hepatocellular carcinoma, and death (Cotter & Rinella, 2020; Targher et al., 2021). NAFLD is a multi-factor and poorly-understood disease and is generally prevalent in patients suffering from the metabolic syndrome, such as obesity and type 2 diabetes (Targher et al., 2018). Therefore, the mechanisms and treatment approaches of NAFLD should be well elucidated and established (Loomba & Sanyal, 2013).

Despite its importance, guideline-recommended medications for NAFLD patients are scarce (Paternostro & Trauner, 2022; Targher & Byrne, 2017). According to the European- and American Association for the Study of the Liver, only vitamin E and pioglitazone are recommended for selected NAFLD patients (Paternostro & Trauner, 2022). Other drug options for different NAFLD targets (such as glucagon-like peptide-1 (GLP-1) receptor agonist, thyroid hormone receptor beta agonist, etc.) are under evaluation, but most of them are limited to either

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selected patients or adverse drug reactions (Rinella & Sanyal, 2016; Rotman & Sanyal, 2017). Apart from medication, dietary interventions could be the alternative approaches for NAFLD prevention and alleviation as dietary patterns are highly contributed to the prevalence and progression of NAFLD (Marchesini et al., 2016; Romero-Gomez et al., 2017). The related mechanism behind this can be attributed to the gut microbiota. Emerging research suggests that gut microbiota and its-derived mediators (such as short-chain fatty acids (SCFAs)) can be used as potential biomarkers in the prognosis, treatment and diagnosis of NAFLD based on the gut-liver axis relationship (El Hage et al., 2020; Gill et al., 2018; Han et al., 2023). A high-fat diet (HFD) can induce gut dysbiosis, which can cause NAFLD by negatively affecting hepatic metabolism of lipids and cholesterol via modulating gut microbiota and their metabolites (Doulberis et al., 2017; Mouzaki et al., 2013). Therefore, targeting gut microbiota via dietary intervention may be an effective and safe strategy for the prevention and mitigation of NAFLD.

As a natural bioactive compound and dietary supplement, resveratrol (RSV) has exhibited a broad biological activity, including cardioprotection, anti-cancer effects, antioxidant and anti-inflammatory properties, regulation of glycolipid balance, and inhibition of fat accumulation (Jardim et al., 2018; Khattar et al., 2022; Singh et al., 2019). Several studies have shown that RSV may benefit NAFLD treatment (Choi et al., 2014; Xin et al., 2013; Zhang et al., 2015), but its strengths and underlying mechanisms for NAFLD therapeutic potentials remain largely elusive due to its poor bioavailability, characterized by poor water solubility and chemical instability (Chachay et al., 2014; Wan et al., 2018). Therefore, further investigation on improving the bioavailability of RSV and its therapeutic effects on NAFLD, along with the related mechanisms, is warranted.

In this study, we conducted prevention and intervention trials to investigate the effects of RSV in NAFLD mice. The NAFLD model was established by inducing mice with an HFD. RSV was encapsulated by Hydroxypropyl-beta-Cyclodextrin (HBC) to enhance its water solubility. Both prevention and intervention trials were performed through intragastric administration of RSV and HBC-RSV. Subsequently, we evaluated body weight, viscera weight, liver and serum biochemical indexes, relative mRNA expression levels, fecal SCFAs concentrations, and sequenced the gut flora of the mice.

2. Materials and methods

2.1. Chemicals

RSV, HBC, absolute ethanol and standard acetic acid, propionic acid were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and Sigma-Aldrich (St. Louis, MO, USA). Chromatography grade methanol and acetonitrile were provided by Merck (Darmstadt, Germany), and other reagents used were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Drug entrapment efficiency of HBC-RSV

The calculation of the entrapment efficiency (EE (%)) of HBC-RSV was based on the previous method with some modifications (Zhu et al., 2022). In short, RSV was embedded into HBC at a mass ratio of 1:10, and then the HBC-RSV solution was freeze dried by LGJ-10N freeze dryer from Beijing Yaxing Yike Technology Development Co., Ltd. (Beijing, China). For the sake of determining the drug EE (%) of HBC-RSV, a certain amount of RSV was weighed and dissolved into a certain volume of absolute ethanol, preparing 40, 20, 10, 5 μg/mL solutions with normal saline, and then the absorbance value at 306 nm was detected with a BioTek microplate reader (Vermont, America). The standard curve (Fig. S1) was drawn according to the absorbance, and RSV was dissolved in physiological saline to measure the absorbance. The EE (%) was calculated according to the following formula, and final EE (%) of RSV was 86%. The freeze-dried RSV powder was collected and stored at -20 °C for the following of experiment.

$$\mathrm{EE}\ (\%) = \frac{\text{theoretical amount of RSV} - \text{unembedded RSV}}{\text{theorerical amount of RSV}} \times 100\%$$

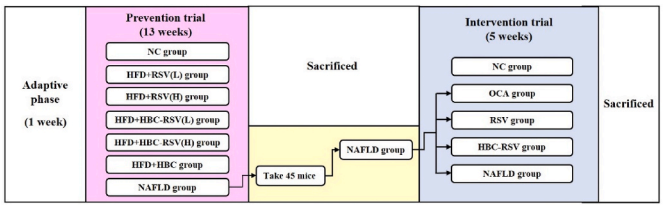

Fig. 1. Schematic diagram of the prevention and intervention trial, respectly. For the prevention trial, mice were divided into seven groups and subjected to different trials for 13 weeks (NC group: normal feed; HFD + RSV (L): 20 mg/kg/d RSV; HFD + RSV (H): 100 mg/kg/d RSV; HFD + HBC-RSV (L): HBC-embedded 20 mg/kg/d RSV; HFD + HBC-RSV (H): HBC-embedded 100 mg/kg/d RSV; HFD-HBC: the same amount of HBC used for embedding RSV). For the intervention trial, NAFLD mice were divided into four groups for 5 weeks, All of them were fed normally and used for different treatments (OCA: 30 mg/kg/d drug OCA; RSV: 100 mg/kg/d RSV; HBC-RSV: HBC-embedded 100 mg/kg/d RSV; NAFLD: normal saline).

2.3. Dosage information

It was based on a study conducted in animals, which demonstrated that RSV effectively alleviated the development of NAFLD at a dose of 100 mg/kg/d (Heeboll et al., 2015). In this study, therefore, RSV was given by gavage to 6-week age mice at 0, 20, and 100 mg/kg/d. According to the 86% EE of RSV mentioned above, the 122 mg/mL HBC-RSV (L), 130 mg/mL HBC-RSV (H) and 120 mg/mL HBC solution were prepared with normal saline and the final intragastric dose of HBC reached 1.2 g/kg/d.

2.4. Animal treatments

All experimental protocols employed herein were approved by the Committee on the Care of Laboratory Animal Resources, College of Biological Science and Engineering, Fuzhou University (2019-SG-007). Male SPF C57BL/6 mice (age 6 weeks, 15–18 g) were purchased from Shanghai Ling Cheong Biotechnology Co., Ltd. (Shanghai, China). Mice were kept in a room with a controlled temperature of 23 ± 1 °C and humidity of 50 ± 1%, under a reversed 12 h light/dark cycle (lights on 09:00–21:00 h) with ad libitum access to food and water, except during the experiments. Animals were first acclimated to the lab for 1 week before the start of the experiments.

In this study, mice were fed an HFD (60 kcal% fat; D12492; Research Diets, New Brunswick, USA) to establish NAFLD models (Dai et al., 2022). As shown in Fig. 1, mice were randomly divided into seven groups for a 13-week of prevention trial. Mice fed with a normal diet comprised the normal control (NC) group, while those fed with a 60% high-fat diet (HFD) served as the NAFLD model group. The remaining mice were also fed with a 60% HFD and given various substances by gavage. At the end of the 13th week, three mice were randomly selected from the NAFLD model group. Liver samples were stained with H&E to confirm the establishment of NAFLD, while the other model mice were subjected to an additional 5-week intervention trial. Notably, during the intervention experiment, obeticholic acid (OCA) was utilized as the positive drug control (Lin et al., 2022), administered via gavage at a dose of 30 mg/kg/d. Throughout both the prevention and intervention trials, the mice were weighed weekly, with their weight gain subsequently plotted as a growth curve. Body length was measured at the conclusion of the experiment.

After the mice dying of asphyxia, blood was collected from the abdominal vein. The liver, kidney, spleen, and abdominal fat were removed and weighed, while samples of the rectal, colon, cecum wall,

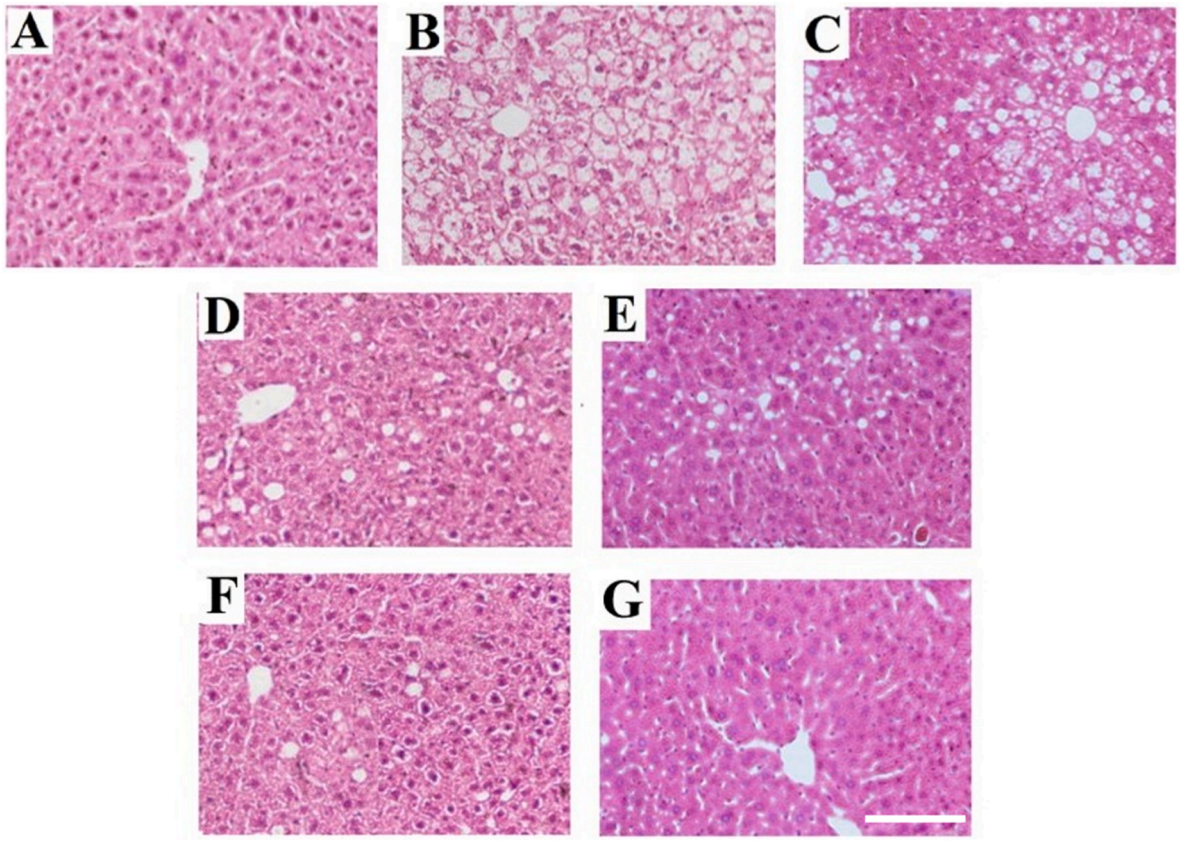

Fig. 2. Effects of RSV and HBC-RSV on hepatic steatosis in mice fed with HFD for 13 weeks in prevention trial (200 ×). (A) NC group; (B) NAFLD group; (C) HBC lavage group; (D) RSV (L) lavage group; (E) HBC-RSV (L) lavage group; (F) RSV (H) lavage group; (G) HBC-RSV (H) lavage group.

and their contents were also collected (all operations were carried out on the super clean workbench), quickly frozen with liquid nitrogen and stored in the refrigerator at -80 °C for standby.

2.5. Detection of liver biochemical indexes

The processing method of liver tissue was followed as previously (Lan et al., 2021). In brief, 50 mg of liver sample was accurately weighed and placed into a 1.5 mL centrifuge tube. 450 μL of normal saline pre-cooled at 4 °C was added in the ratio of weight (g) to volume (mL) = 1 : 9. The centrifuge tube was inserted into crushed ice and mechanically homogenized at 2500 rpm/min for 10 min. Liver triglyceride and liver cholesterol were detected by triglyceride (TG) kit and total cholesterol (TC) kit purchased from Nanjing Jiancheng Technology Co., Ltd. (Shanghai, China).

2.6. Detection of biochemical indexes in serum

The collected mice abdominal vein blood was centrifuged at 3000 rpm/min at room temperature for 10 min, and the supernatant was collected and packed for later use (Li et al., 2022). Serum high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were detected by kit purchased from Nanjing Jiancheng Technology Co., Ltd. (Shanghai, China).

2.7. Liver mRNA expression

Total RNA was isolated from mouse liver tissue using spin column animal total RNA purification kit (Sangon Biotech, China) following the manufacturer's instructions. cDNA was synthesized by reverse transcription kit (TransGen Biotech, China). Real-time PCR was performed using TransStart Tip Green qPCR Super Mix (TransGen Biotech, China). Primers (Table S1) were used to creen the mRNA expression of sterol receptor element binding protein 1c (SREBP1c), acetyl-coenzyme A carboxylase (ACC), peroxisome proliferators-activated receptorα (PPARα) and AMP-activated protein kinase (AMPK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control gene, the relative mRNA expression of liver gene was calculated by $2^{-\Delta Ct\Delta Ct}$ method (Latorre et al., 2022).

2.8. Fecal SCFAs detection

Fecal SCFAs were determined by high performance liquid chromatography (HPLC) (Hati et al., 2019) with some modifications. Mice feces were removed, weighed, and dissolved with 1.8 mM hydrochloric acid at 10 μL/mg. After ultrasonic treatment for 2 h, the mixture was centrifuged at 15000 rpm for 10 min at 4 °C, then supernatants were syringe filtered with 0.22 × 10^{-6} M filters (Corning). The concentrations of SCFAs were measured by HPLC (Thermo, MA, USA) with Column Acclaim TM Organic Acid C18 (5 μm, 250 × 4.0 mm) (SN:002227) at a column temperature of 30 °C and wavelength of 210 nm. Samples (20 μL) were injected into the HPLC, the autosampler was set at a temperature of 6 °C.

2.9. 16S rRNA sequencing of gut microbiota

Fecal DNA was extracted with EasyPure Stool Genomic DNA Kit (TransGen Biotech, China). 2 μL DNA sample was took for determining the absorbance of sample to ensure that the OD 260/280 ratio range was between 1.8 and 2.0. Then DNA samples were used for the analysis of fecal microbiota using Illumina HiSeq technology at Biomarker Technologies Co., Ltd. (Beijing, China). The V3–V4 region of the bacteria 16S

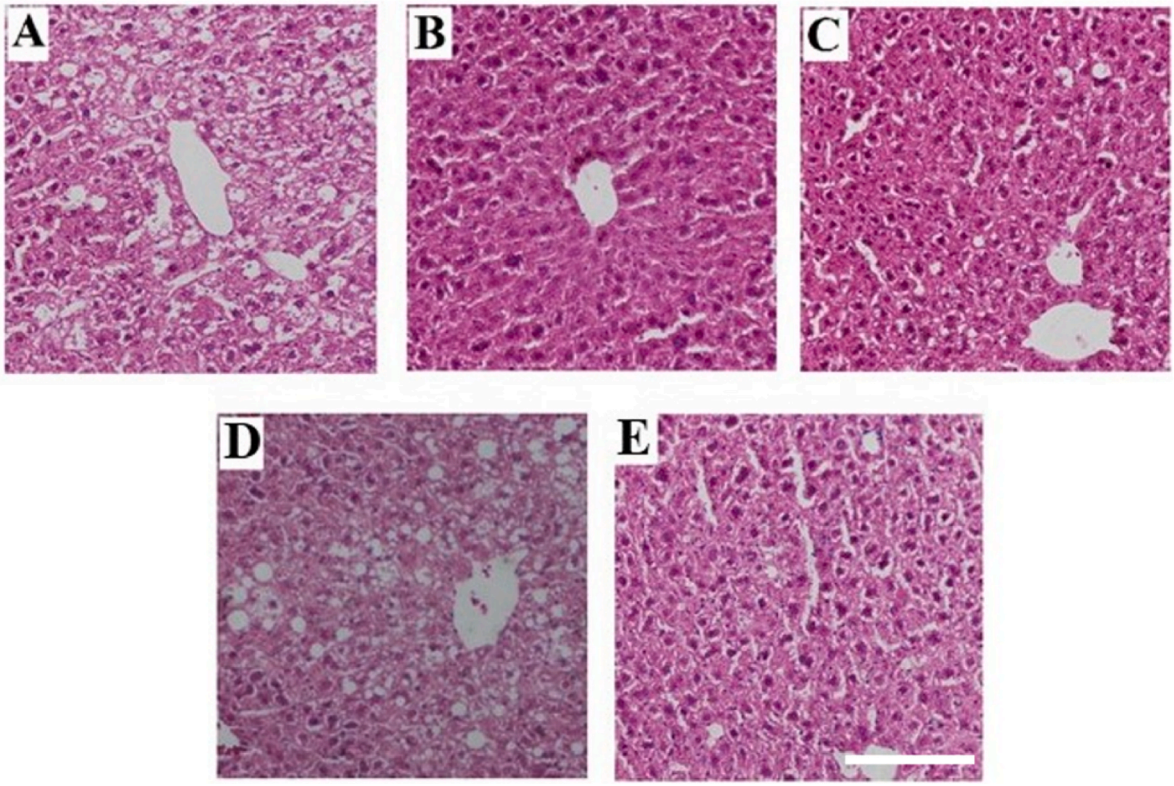

Fig. 3. Liver pathological H&E staining of mice with NAFLD treated with RSV and HBC-RSV for 5 weeks (original magnification × 200). (A) NAFLD group; (B) OCA drug treatment group; (C) RSV lavage group; (D) HBC lavage group; (E) HBC-RSV lavage group.

ribosomal RNA genes was amplified by PCR using primers designed according to the conserved region with an adapter at the end. The amplified products were purified, quantified and homogenized to form the sequencing library. After passing the quality inspection, Shanghai Meiji Biomedical Technology Co., Ltd. (Shanghai, China) was commissioned to conduct high throughput sequencing. Flash V1.2.7 software was used to generate raw labels from the high-throughput sequencing readings. Original labels were processed using Trimomatic V0.33 and UCHIME V4.2 softwares to obtain valid labels. Valid tags were clustered by QIIME (version 1.8.0) software to generate operational taxonomic units (OTUs) at 97% similarity level. According to the representative sequence of species corresponding to each OTU, the microbial community composition in each sample at different levels was calculated by comparison with the microbial database. The α-diversity indexes including Shannon index, Simpson index, Ace and Chao indexes were evaluated using Mothur (version v.1.30) software.

2.10. Statistical analysis

The statistical analysis was performed using SPSS statistical package (SPSS, Chicago, IL, USA) (El-Hadary et al., 2023). Student's *t*-test was applied for comparisons between averages of two samples, and the equality of variances of was assessed by Levene's test. If equal variances were not assumed, the nonparametric Mann-Whitney *U* test was applied. *p*-value <0.05 was used as the threshold of statistical significance.

3. Results

3.1. HBC embedding improved the water solubility of RSV

RSV was encapsulated in HBC to enhance its water solubility and pharmacological potential. Based on the standard curve (Fig. S1), the encapsulation efficiency (EE) of HBC-embedded RSV (H) was 86%, and the water solubility was increased by approximately 250-fold (Table S2).

3.2. Effects of RSV and HBC-RSV on body weight, visceral weight, pathological change and Lee's index in mice fed with HFD

When the mice were fed with an HFD for a duration of 13 weeks, we observed the presence of diffuse bullous steatosis in the liver tissue of the NAFLD mice (Fig. 1B). This finding confirmed the successful establishment of the NAFLD mouse model. Additionally, we measured Lee's index, which serveed as a biomarker for assessing the degree of obesity and found that NAFLD mice exhibited significantly higher levels of Lee's index compared to the control group (Table S3). Furthermore, upon combining the results of pathological changes and Lee's index, we observed that higher concentrations of RSV and HBC-RSV significantly reduced the degree of obesity induced by HFD in mice. Notably, the HBC-RSV group exhibited more pronounced beneficial effects compared to the RSV group in both the prevention and intervention trials (Fig. 2-3, and Tables S3–4).

In the analysis of common physiological indices (Figs. S2A–J), it was found that the HFD significantly increased the body weight and visceral weight, including the liver, kidney, spleen, and abdominal fat, in mice. However, the administration of RSV or HBC-RSV in both prevention and intervention trials effectively inhibited the weight gain induced by HFD. Notably, the higher concentration of HBC-RSV demonstrated, the greater efficacy in promoting weight loss.

3.3. Effects of RSV and HBC-RSV on lipids in mice fed with HFD

In the prevention experiment, relative to the NAFLD group, the higher concentration of HBC-RSV significantly inhibited the level of TG and TC caused by HFD, while simultaneously increased the level of HDL-C and reduced the content of LDL-C in the serum of mice compared to

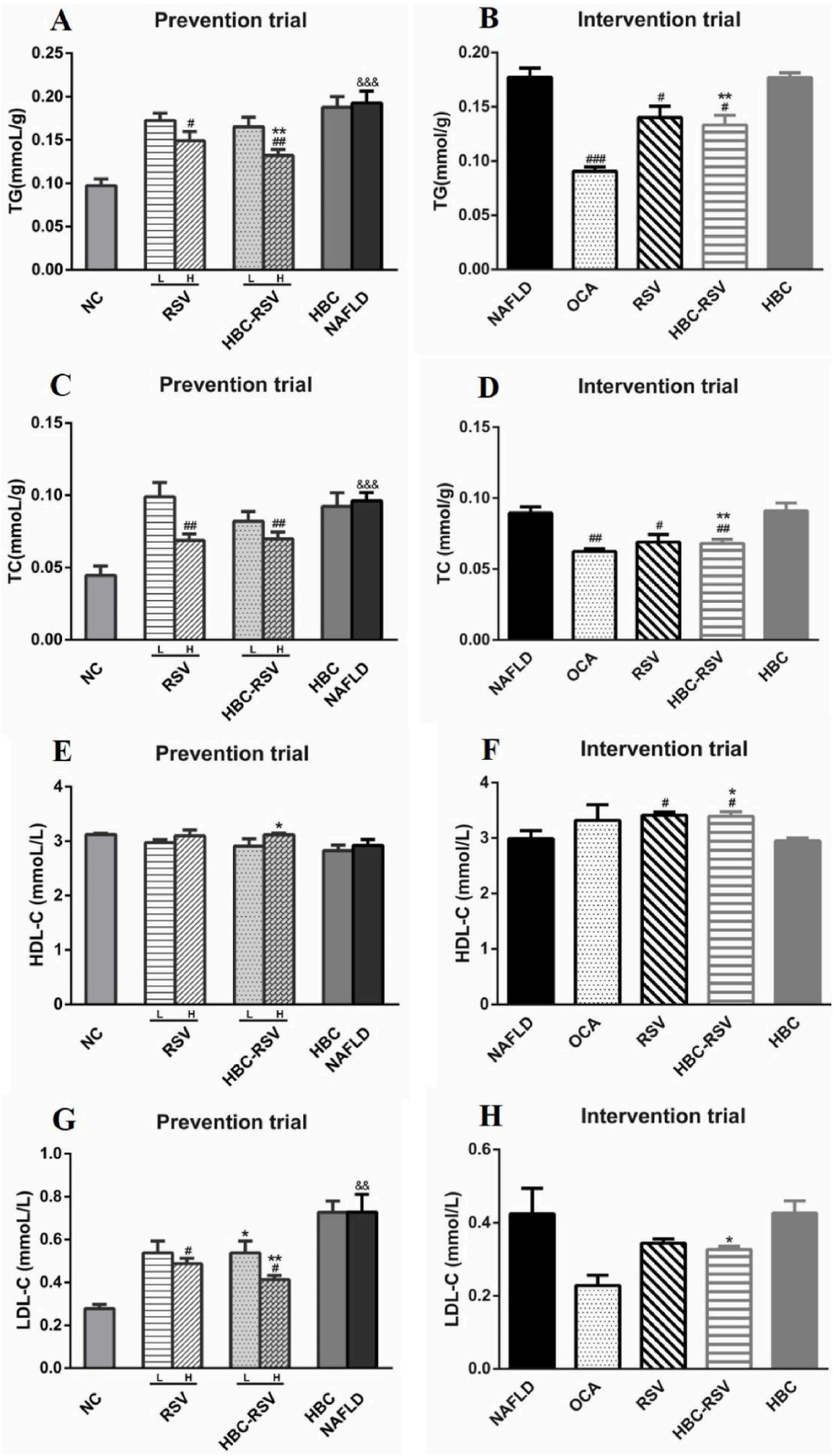

Fig. 4. Effects of daily intake of RSV and HBC-RSV on lipid content in mice fed with HFD. A, B, C and D: Changes in TG and TC levels in livers. E, F, G and H: Changes in HDL-C and LDL-C in serums. &&: $p < 0.01$, &&&: $p < 0.001$ based on the Student's *t*-test, and significantly different from the NC group. #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.001$ based on the Student's *t*-test, and significantly different from the NAFLD group. *: $p < 0.05$, **: $p < 0.05$ based on the Student's *t*-test, and significantly different from the HBC group. Data are shown as mean ± SEM. The number of mice in each group is six.

the NAFLD group (Fig. 4A, C, E, G). In the intervention experiment, as a drug for the treatment of NAFLD, OCA significantly reduced the content of TG and TC in the liver of NAFLD mice but had no significant effect on the synthesis of HDL-C and the decrease of LDL-C. HBC-RSV could still reduce the contents of TG, TC, and LDL-C and increase the content of HDL-C. Similar results were also found in the intragastric administration of a single RSV, except for LDL-C (Fig. 4B, D, F, H).

3.4. *Effects of RSV and HBC-RSV on the gut microbiota in mice fed with HFD*

By sequencing the 16S rRNA of feces in mice, we observed significant

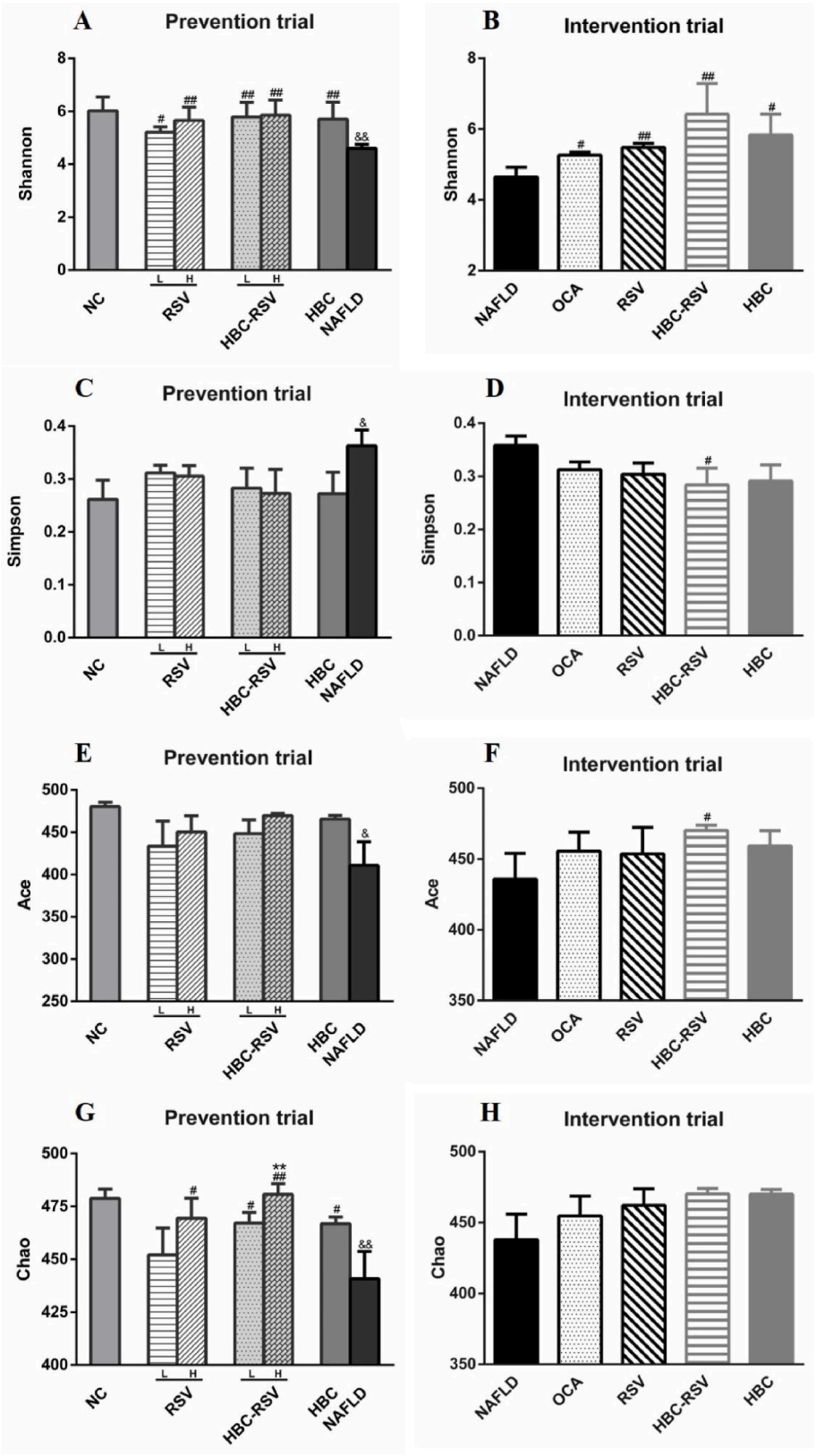

Fig. 5. Effects of RSV and HBC-RSV on community diversity and richness of gut microbiota in mice fed with HFD. A, B, C and D: The analysis of species richness based on Shannon and Simpson indexes. E, F, G and H: The analysis of species richness based on Ace and Chao indexes. &: $p < 0.05$, &&: $p < 0.01$ based on the Student's *t*-test, and significantly different from the NC group. #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.001$ based on the Student's *t*-test, and significantly different from the NAFLD group. *: $p < 0.05$, **: $p < 0.01$ based on the Student's *t*-test, and significantly different from the HBC group. δ: $p < 0.05$ based on the Student's *t*-test, and significantly different from the RSV group. Data are shown as mean ± SEM. The number of mice in each group is six.

differences in the Shannon and Simpson indexes, which represented the diversity of gut microbiota, as well as the Ace and Chao indexes, which represented species richness, between the NAFLD group and the control group (Fig. 5A, C, E and G). In the prevention experiment, daily intake of RSV and HBC-RSV effectively prevented the significant decrease of the Shannon and Chao indexes in HFD-fed mice (Fig. 5A and G), while no significant change was observed in the Simpson and Ace indexes (Fig. 5C and E). In the intervention experiment, intragastric administration of RSV and HBC-RSV significantly increased the value of the Shannon index (Fig. 5B). Moreover, it was observed that only the intervention

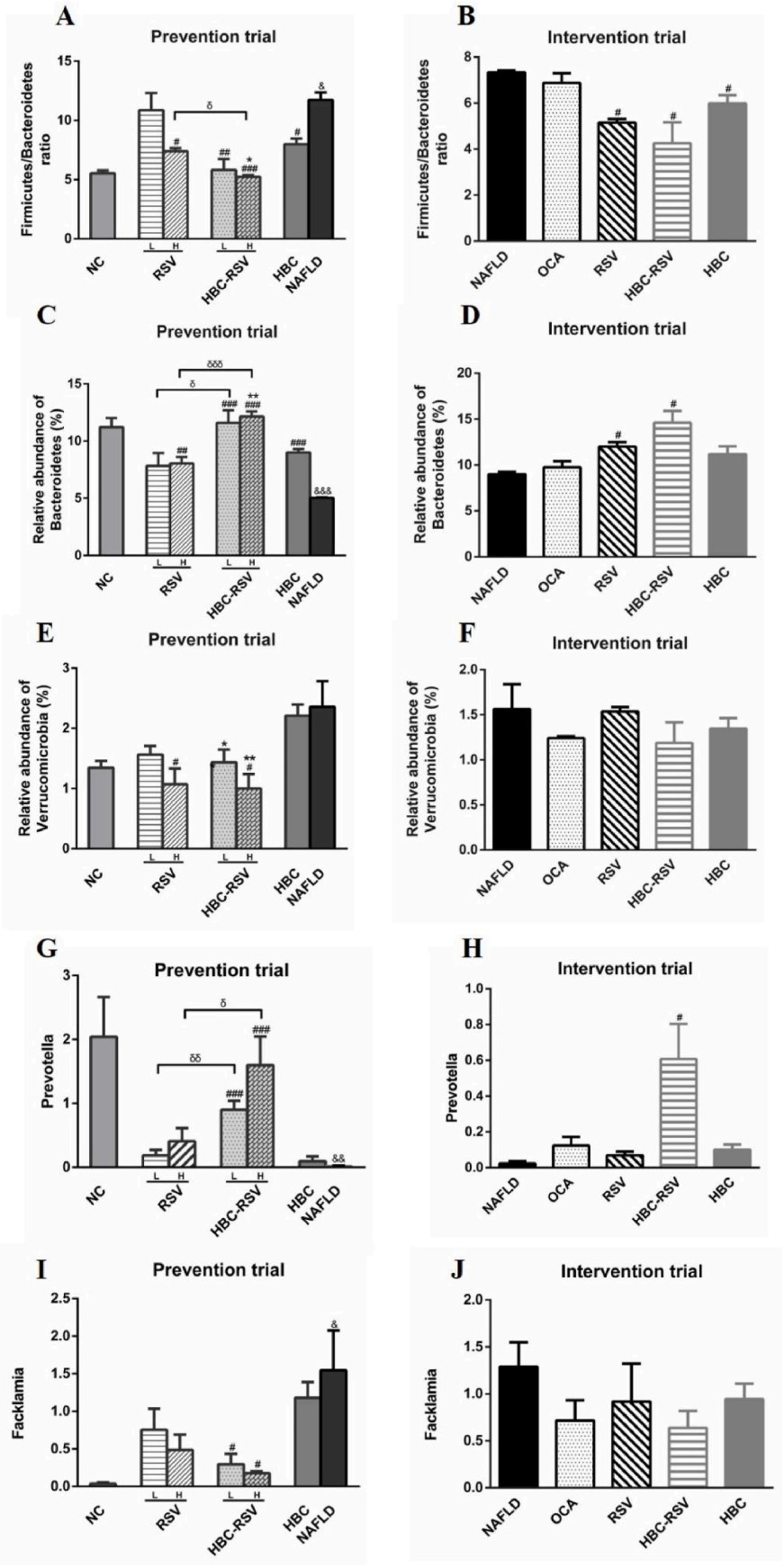

Fig. 6. Effects of RSV and HBC-RSV on the gut microbiota at the phylum and genus levels in mice fed with HFD. A and B: The ratio of *Firmicutes/Bacteroidete.* C, D, E and F: The relative abundances of *Bacteroidetes* and *Verrucomicrobia* at phylum level. G, H, I and J: The relative abundances of *Prevotella* and *Facklamia* at the genus level. &: $p < 0.05$, &&: $p < 0.01$ and &&&: $p < 0.001$ based on the Student's *t*-test, and significantly different from the NC group. #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.001$ based on the Student's *t*-test, and significantly different from the NAFLD group. *: $p < 0.05$, **: $p < 0.01$ based on the Student's *t*-test, and significantly different from the HBC group. δ: $p < 0.05$, $\delta\delta$: $p < 0.01$ and $\delta\delta\delta$: $p < 0.001$ based on the Student's *t*-test, and significantly different from the RSV group. Data are shown as mean ± SEM. The number of mice in each group is six.

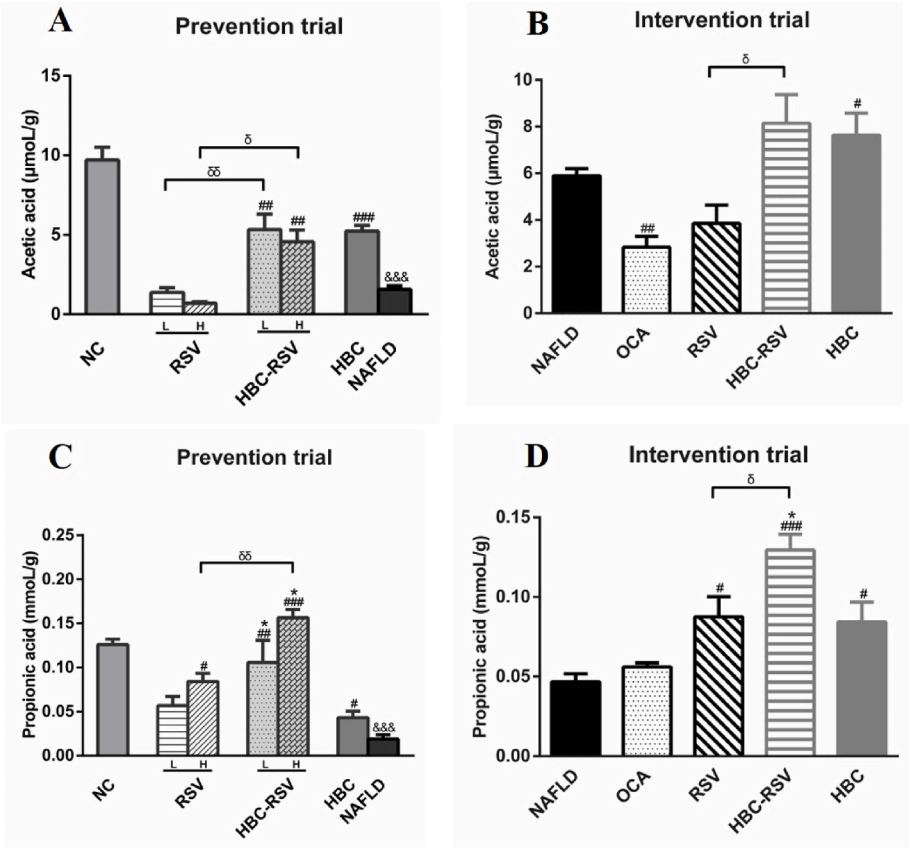

Fig. 7. Effects of RSV and HBC-RSV on fecal SCFAs concentrations in mice fed with HFD. A, B, C and D: Concentrations of acetic acid and propionic acid in mice feces. &&&: $p < 0.001$ based on the Student's *t*-test, and significantly different from the NC group. #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.001$ based on the Student's *t*-test, and significantly different from the NAFLD group. *: $p < 0.05$ based on the Student's *t*-test, and significantly different from the HBC group. δ: $p < 0.05$, δδ: $p < 0.01$ based on the Student's *t*-test, and significantly different from the RSV group. Data are shown as mean ± SEM. The number of mice in each group is six.

with HBC-RSV significantly improved the Simpson and Ace indexes in NAFLD mice (Fig. 5D and F).

The histogram depicting the percentage distribution of gut microbiota at the phylum level in NAFLD mice with treatment of RSV and HBC-RSV is presented in Fig. S3, and the statistical chart of *Firmicutes/Bacteroidetes* ratio was obtained after quantification. As shown in Fig. 6A, the *Firmicutes/Bacteroidetes* ratio in NAFLD mice was significantly higher compared to the control group. The administration of RSV (H), HBC, and two concentrations of HBC-RSV effectively prevented the increase in the *Firmicutes/Bacteroidetes* ratio in HFD-fed mice. Furthermore, the preventive treatment of HBC-RSV (H) exhibited a lower ratio compared to the RSV (H) group. In the intervention experiment, RSV, HBC, and HBC-RSV demonstrated a decreasing effect on the *Firmicutes/Bacteroidetes* ratio (Fig. 6B).

In the presence of HFD, a reduction in the abundance of *Bacteroidetes* and an increase in *Verrucomicrobia* were observed at the phylum level in the intestinal tract of mice. However, oral administration of HFD along with RSV and HBC-RSV, particularly at higher concentrations of RSV, effectively prevented the HFD-induced changes in *Bacteroidetes* (Fig. 6C) and *Verrucomicrobia* microflora (Fig. 6E). In the intervention experiment, on the other hand, RSV and HBC-RSV only increased the relative abundance of *Bacteroidetes*, but did not reduce the relative abundance of *Verrucomicrobia* microflora in the mice intestine (Fig. 6F).

At the genus level, HFD significantly decreased the relative abundance of *Prevotella* and increased the relative abundance of *Facklamia* compared to the control group. Further analysis revealed that HBC-embedded RSV, at both low and high concentrations, effectively prevented the changes in the relative abundance of *Prevotella* and *Facklamia*, which was not observed in the single RSV group (Fig. 6G and I). However, in the intervention experiment, only HBC-RSV demonstrated a significant improvement in the increment of *Prevotella* (Fig. 6H).

3.5. Effects of RSV and HBC-RSV on fecal SCFAs in mice fed with HFD

The SCFAs produced by the gut microbiota include acetic acid, propionic acid and butyric acid. Analysis of fecal SCFAs in mice revealed that HFD significantly reduced the production of SCFAs in the intestinal tract. In the prevention experiment, neither high nor low concentrations of RSV increased the concentration of acetic acid in the intestinal tract of mice (Fig. 7A). However, high concentrations of RSV and HBC-embedded RSV significantly increased the concentration of propionic acid, with the HBC-RSV group demonstrating more pronounced beneficial effects compared to the single RSV group (Fig. 7C). Interestingly, HBC alone also increased the level of acetic acid compared to the NAFLD mice. Similar results were observed in the intervention experiment, where RSV, HBC-RSV, and HBC interventions all increased the content of propionic acid in the mouse intestine, with HBC-RSV showing the most significant promotion effect (Fig. 7B, D).

3.6. Effects of RSV and HBC-RSV on mRNA expression of hepatic lipid metabolism-related genes in mice fed with HFD

As depicted in Fig. 8A–H, the consumption of HFD resulted in abnormal mRNA expressions of liver lipid metabolism-related genes, including SREBP1c, ACC, PPARα, and AMPK in mice. In both the prevention and intervention trials, daily administration of RSV and HBC-RSV effectively prevented the HFD-induced increase in mRNA expression levels of SREBP1c and ACC, as well as the decrease in mRNA expression levels of PPARα and AMPK when compared to NAFLD mice. Moreover, we observed that HBC-embedded RSV, particularly at higher concentrations, exhibited a more pronounced efficacy than non-embedded RSV, especially regarding the expression level of ACC mRNA in the prevention trial.

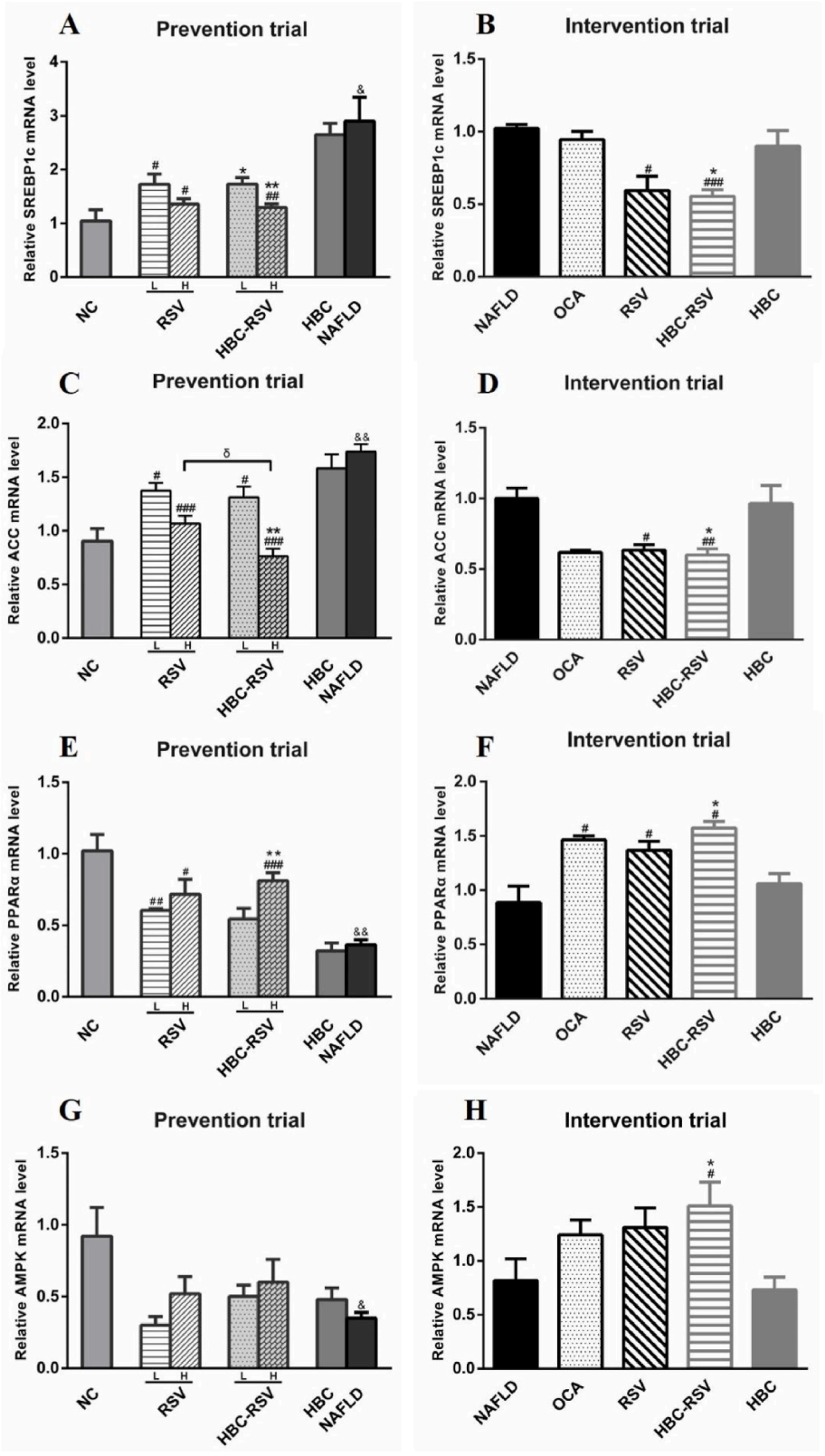

Fig. 8. Effects of RSV and HBC-RSV on lipid metabolism-related mRNA expression in liver of mice fed with HFD. A, B, C and D: mRNA expression of SPEBP1c and ACC genes related to liver lipid metabolism. E, F, G and H: mRNA expression of PPARα and AMPK genes related to liver lipid metabolism. *&*: $p < 0.05$, *&&*: $p < 0.01$ based on the Student's *t*-test, and significantly different from the NC group. *#*: $p < 0.05$, *##*: $p < 0.01$ and *###*: $p < 0.001$ based on the Student's *t*-test, and significantly different from the NAFLD group. ***: $p < 0.05$, ****: $p < 0.05$ based on the Student's *t*-test, and significantly different from the HBC group. *δ*: $p < 0.05$ based on the Student's *t*-test, and significantly different from the RSV group. Data are shown as mean ± SEM. The number of mice in each group is six.

4. Discussion

Understanding the pathogenesis of NAFLD is crucial due to the lack of safe and effective medical treatments available (Liang et al., 2019). Emerging research suggests that targeting the gut microbiota may be a potential NAFLD treatment strategy based on the gut-liver axis relationship (Compare et al., 2012; El Hage et al., 2020; Han et al., 2023). Additionally, changes in dietary patterns, such as long-term adherence to vegetarian or Mediterranean diets, have been shown to prevent or alleviate NAFLD by optimizing the gut microbiota composition (Feng et al., 2017; Leung et al., 2016; Zhang et al., 2020).

However, to strengthen the rationale for diet intervention, further

evidence is required. Specific studies are needed to investigate the molecular mechanisms underlying the benefits of dietary patterns and their impact on optimizing the gut microbiota composition in order to prevent or alleviate NAFLD. On this foundation, it is worthwhile to explore whether NAFLD prevention can be achieved through the supplementation of diets rich in bioactive compounds.

As a natural polyphenolic nutraceutical, RSV is considered to exhibiting multiple pharmacological activities (Singh et al., 2019). However, it is still controversial whether RSV can benefit NAFLD prevention and alleviation (Chachay et al., 2014). In our study, we used the HBC to increase the water solubility of RSV and verified the better preventive and therapeutic effects of HBC-embedded RSV, including improving liver steatosis, obesity, and aberrant lipid metabolism brought on by HFD.

Further analysis of our data revealed that HFD significantly reduced the α-diversity of the gut microbiota in mice and that RSV supplementation might mitigate the negative effects. Specifically, previous studies have demonstrated that obese individuals with NAFLD typically exhibit a higher abundance of *Firmicutes* and a lower proportion of *Bacteroidetes* at the phylum level (Song et al., 2020), and we observed that RSV could significantly prevent an increase in the ratio of *Firmicutes/Bacteroidetes*. Additionally, HFD increased the relative abundance of *Verrucomicrobia*, which was similar to previous studies (Aron-Wisnewsky et al., 2020; Carmody et al., 2015), while RSV inhibited this change. *Verrucomicrobia* has been reported to disturb host mucus homeostasis and aggravate intestinal inflammation caused by *Salmonella* in sterile mice (Ganesh et al., 2013).

At the genus level, RSV increased the level of *Prevotella* and decreased the level of *Facklamia* in HFD-fed mice. *Prevotella* can improve glucose metabolism, while *Facklamia* has been reported as a human pathogen (Ohtsu et al., 2019; Wang et al., 2021). Therefore, RSV, especially HBC-embedded RSV, resulting in beneficial changes (increasingbeneficial bacteria and decreasing potentially pathogenic bacteria) in the gut microbiota might contribute to reducing the risk of NAFLD occurrence in the prevention group.

The proportion and levels of SCFAs are closely associated with the composition of the gut microbiota (Tan et al., 2014). Our findings indicated that RSV supplementation could greatly prevent the decrease in propionic acid caused by HFD. Propionic acid, mainly produced by *Bacteroidetes* in the gut, plays an important role in obesity (Den et al., 2013; Ke et al., 2019). The fact about propionic acid preventing obesity is that it could inhibit the synthesis of cholesterol and fatty acids in the liver of mice (Al-Lahham & Rezaee, 2019). Moreover, propionic acid could also increase the secretion of casein peptide YY (PYY) and GLP-1, and the former might be involved in the transmission of satiety signals and the latter might be involved in the inhibition of food intake (Psichas et al., 2015). To summarize, these results indicated that HBC-embedded RSV rather than RSV alone exerted beneficial effects on gut microbiota composition and SCFAs production, which might contribute to the prevention and alleviation of NAFLD. Besides, HFD could also lead to a decrease in acetic acid, and the downregulation was stopped by HBC rather than RSV. The acetic acid has been reported to improve glucose tolerance and to have anti-inflammatory effects (Urtasun et al., 2020; Wang et al., 2018). So it was suggested that HBC probably has a synergistic effect on NAFLD mice treated with HBC-embedded RSV. HBC is a modified oligosaccharide that can regulate intestinal homeostasis and promote the growth of intestinal probiotics (Ren et al., 2022). However, it could be intuitively seen from the results of our H&E liver pathological section and other negative data that HBC itself cannot prevent NAFLD. On the other hand, many studies proved that SCFAs including propionic acid and acetic acid, could promote gut integrity and homeostasis by promoting the growth of symbionts and inhibiting the growth of pathobionts (Laparra & Sanz, 2010; Tan et al., 2014; Wang et al., 2018). Thus, taken together, intragastric administration of RSV, especially HBC-RSV, had a better preventive effect by increasing the relative abundance of *Bacteroidetes* and the levels of SCFAs in HFD-induced NAFLD mice.

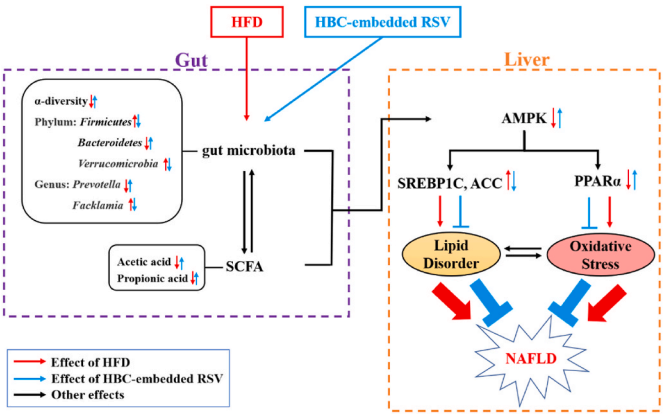

Fig. 9. Potential mechanism of HBC-embedded RSV on preventing NAFLD.

In accordance with a previous study (Choi et al., 2014), our results suggested that long-term feeding of HFD impaired the AMPK signaling pathway. AMPK plays a key regulatory role in lipid metabolism, which is one of the most important molecular mechanisms involved in NAFLD (Ren et al., 2020). Moreover, our study revealed that RSV was capable of activating AMPK, and this activation mechanism may be related to RSV-regulated SCFAs, which can directly impact AMPK activation (Den et al., 2013; Song et al., 2019). The activated AMPK can in turn down-regulated SREBP1c and ACC while up-regulating PPARα, ultimately leading to inhibition of lipogenesis and promotion of fatty acid oxidation in the liver (Liu et al., 2020). To summarize, the evidence was conjectured that RSV might be beneficial to the liver lipid metabolism by ameliorating the gut microbiota and raising the levels of SCFAs to activate AMPK (including down-regulaing SREBP1c and ACC and up-regulating PPARα), thereby reducing lipid disorder and oxidative stress to prevent NAFLD (Fig. 9).

Besides, it should be mentioned that, in order to gather more detailed information, particularly in humans, future studies are required to fully evaluate the potential of high-bioavailability RSV supplementation as a dietary prevention for NAFLD.

In general, our findings suggested that HBC-embedded RSV supplementation can prevent and alleviate NAFLD by enhancing the regulation of gut microbial composition, such as selective modulation of beneficial bacteria and increasing SCFAs concentrations, triggering the AMPK signal pathway related to lipid metabolism. These results provided new evidence to elucidate the mechanisms involved in the interactions between HBC-embedded RSV, gut microbiota, and NAFLD, and suggested that HBC-embedded RSV may be a promising and relatively safe method for the prevention of NAFLD.

5. Conclusion

The results of this study evidenced that HBC-embedded RSV supplementation can prevent and alleviate NAFLD by enhancing the regulation of gut microbial composition, such as selective modulation of beneficial bacteria and increasing SCFAs concentrations, triggering the AMPK signal pathway related to lipid metabolism. These findings revealed that HBC-embedded RSV may be a promising and generally secure strategy for the prevention of NAFLD and offered new information on the mechanisms underlying the interactions between gut microbiota, HBC-embedded RSV, and NAFLD.

Author statement

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Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Data availability

Data will be made available on request.

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Abbreviations

NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
GLP-1	glucagon-like peptide-1
HFD	high fat diet
SCFAs	short chain fatty acids
RSV	resveratrol
HBC	Hydroxypropyl-beta-Cyclodextrin
EE	entrapment efficiency
OCA	obeticholic acid
TG	triglyceride
TC	total cholesterol
LDL-C	low-density lipoprotein cholesterol
HDL-C	high-density lipoprotein cholesterol
SREBP1c	sterol receptor element binding protein 1c
ACC	acetyl-coenzyme A carboxylase
PPARα	peroxisome proliferators-activated receptorα
AMPK	AMP-activated protein kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PYY	peptide YY

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102907.

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