



## Research Paper

## Beneficial effect of vinegar consumption associated with regulating gut microbiome and metabolome

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## ABSTRACT

Vinegar is used as fermented condiment and functional food worldwide. Vinegar contains many nutrients and bioactive components, which exhibits health benefits. In this study, the potential effects of Shanxi aged vinegar (SAV) on gut microbiome and metabolome were explored in normal mice. The levels of inflammatory factors were significantly decreased in SAV-treated mice. Immunoglobulin, NK cells and CD20 expression were significantly increased after SAV administration. In addition, SAV intake altered gut microbiota structure by up-regulating *Verrucomicrobia*, *Akkermansia*, *Hungatella* and *Alistipes*, and down-regulating Firmicutes, *Lachnospiraceae\_NK4A136\_group* and *Oscillibacter*. The differential metabolites were mainly included amino acids, carbohydrates and bile acids. Furthermore, after SAV intake, *Verrucomicrobia*, and *Akkermansia* closely impacted the related gut metabolites. These alterations of gut microbiota-related metabolism further modulated some immunoregulatory and inflammatory factors, and confer potential health benefits. Our results imply that vinegar consumption has beneficial effects on regulating gut microbiome and metabolome.

## 1. Introduction

Vinegar is a traditional product that has been fermented with history over 3000 years worldwide. Vinegar is not only a kind of acid seasoning, but also a functional food. Previous studies have found that vinegar can prevent diseases including obesity (Kondo et al., 2009), liver damage (Xia et al., 2020a), metabolic disorders (Ousaaid et al., 2020) and cancer (Baba et al., 2013). Vinegar contains many nutritional and bioactive compounds including amino acids, carbohydrates, polyphenols, and melanoidins, which play a beneficial role on body health (Xia et al., 2018a; Kandyliis et al., 2021 & Ousaaid et al., 2022).

Gut microbiome play a critical role in human digestion, which can break down complex carbohydrates, proteins, and other components (Oliphant and Allen-Vercoe, 2019). Some dietary substances with high molecular weight are not absorbed in intestine. These substances interact with gut microbiota can alter intestinal microbiome composition and function, and then affect gut metabolism and health of the body (Scott et al., 2013; Cianciosi et al., 2020; Li et al., 2021; Zhang et al., 2021a). It has been reported that about 90–95% of dietary polyphenols with high molecular weight reach the colon in a primary form, which are degraded into low-molecular-weight metabolites by gut microbiota (Higbee et al., 2022; Catalkaya et al., 2020; Prasain and Barnes, 2020). Polyphenols from food including cocoa, tea, and fruits interplay with gut

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**Abbreviations:**

AAPA	amino acids peptides and analogues	IL-12	interleukin-12
AA	acetic acid	IL-17	interleukin-17
ALT	alanine aminotransferase	INF- $\gamma$	interferon-gama
AST	aspartate aminotransferase	LDL-C	low-density lipoprotein-cholesterol
BAAD	bile acids alcohols and derivatives	NK	natural killer
CCC	carbohydrates and carbohydrate conjugates	OPLS-DA	orthogonal partial least squares-discriminant analysis
F/B	Firmicutes/Bacteroidetes	OTUs	operational taxonomic units
GAE	gallic acid equivalents	PCA	principal component analysis
HDL-C	high-density lipoprotein-cholesterol	RE	rutin equivalents
IgA	immunoglobulin A	SAV	Shanxi aged vinegar
IgG	immunoglobulin G	sIgA	secretory immunoglobulin A
IL-1 $\beta$	interleukin-1 $\beta$	TC	total cholesterol
IL-2	interleukin-2	TG	triglyceride
IL-6	interleukin-6	Th0	T helper 0
IL-10	interleukin-10	TNF- $\alpha$	tumor necrosis factor-alpha
		VIP	variable importance for projection

microbiota, which modulate gut microbiota composition and play an essential part in body physiology and metabolism (Exteberria et al., 2013; Zhang et al., 2021b). Other studies found that melanoidins, as large molecules of human diet, are fermented by gut microbiota, and conversely promote the beneficial bacteria growth and subsequently result in the increase of short-chain fatty acids production (Pérez-Burillo et al., 2020). Moreover, dietary polyphenols and melanoidins can modify and determine gut microbiota composition and gene expression, and even alter the metabolic profile of gut microbiome (Ursell et al., 2014). In addition, it has been reported that non-digestible polysaccharides in dietary has the effects of combating obesity and diabetes by altering gut microbiota and its metabolites, regulating bile acid composition, inhibiting fat absorption and protecting of intestinal barrier (Zhang et al., 2021a; Lin et al., 2021).

Accumulating researches have proved that gut microbiome and metabolome play a considerable role in the bridge of dietary nutrients and body health (Yang et al., 2020; Wang et al., 2020). Recent studies demonstrate that some metabolites which are transformed by gut microbiota have effects on intestinal and immune homeostasis, energy-metabolism, and inflammation response (Ursell et al., 2014; Zhang et al., 2021c). The metabolites, as chemical messengers, regulate host immune response and physiological metabolism, play a bridge role between gut microbiota and body (Wang et al., 2019). There is a close and multiple relationship among gut microbiome, metabolome and body's health and homeostasis (Rooks and Garrett, 2016).

In vinegar, acetic acid is a major composition, which has the effects of anti-inflammation, anti-obesity, and blood lipid regulation (Beh et al., 2017). A study reported that vinegar and acetic acid prevented ulcerative colitis through suppressing T helper 1 and T helper 17 cell, and maintained gut immune homeostasis (Shen et al., 2016). In addition, vinegar contains some macromolecular bioactive substances such as combined polyphenols, melanoidins and polysaccharides (Xia et al., 2020b). Our previous study have reported that polyphenol-rich vinegar extract improves gut microbiota and immunity, and inhibits inflammation in alcohol-treated mice (Xia et al., 2020a). Li et al. found that melanoidins in vinegar powder exert anti-inflammatory by inhibiting the mRNA expression and the secretion of interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- $\alpha$ ) in ethanol-induced macrophages (Li et al., 2021). Nevertheless, it remains unclear how bioactive compounds in vinegar interact with gut microbiome and metabolome and further affect the body health.

In this study, biochemical parameters, immune and inflammation factors were evaluated in normal mice after Shanxi aged vinegar (SAV) intake. The effects of SAV on gut microbiome and metabolome were investigated *in vivo*. Furthermore, the relationship among body indexes,

gut microbiota, and the metabolites were explored in normal mice. These data may illustrate the mechanisms of SAV intake influencing normal host.

## 2. Materials and methods

### 2.1. Chemicals and reagents

ELISA kits were provided by eBioscience (San Diego, CA, USA). Detection kits of biochemical indexes were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Vinegar samples were chosen SAV products with 8 year-aging time (Shanxi, China). Synthetic vinegar samples were purchased from local supermarkets. The samples were diluted 4 times with sterile water before administration. The constituents of SAV with 8 year-aging time (SAV-8) have been determined in our previous study (Xia, et al., 2018a). Total phenols and flavonoids contents were  $3.732 \pm 0.329$  mg gallic acid equivalents (GAE)/mL and  $3.425 \pm 0.510$  mg rutin equivalents (RE)/mL, respectively. The content of amino acids in SAV-8 was  $9144.77 \pm 2553.17$   $\mu$ g/mL. The content of acetic acid in SAV-8 was  $5.6 \times 10^4 \pm 5.6 \times 10^3$   $\mu$ g/mL, which is the main component in vinegar and occupied 45.70  $\pm$  1.35% to 64.00  $\pm$  2.22% of total organic acids.

### 2.2. Animal experiments

Experiments on mice were executed following animal ethics. Male ICR mice (4-6 week-old, Beijing Vital River Laboratory Animal Technology Co., Ltd, China) were raised in pathogen-free environment provided by the Animal Ethics Committee of Nankai University (SYXK 2019-0001). The mice were randomly divided into three groups (n = 6–10) including normal control (NC) group, acetic acid (AA) group and SAV group. NC group were orally administrated with distilled water, AA group were treated with synthetic vinegar (2.5 mL/kg b. w.), and SAV group was given with SAV (2.5 mL/kg b. w.) by gavage for 8 weeks (Xia et al., 2018b). They were allowed free access to standard diet and water. In the end, the mice were anesthetized with sodium pentobarbital (50 mg/kg, i. p.) and euthanized by cervical dislocation. Fecal samples were obtained in the penultimate day and placed in  $-80$  °C. In the end, spleen and intestine samples were immediately dissected and placed in  $-80$  °C.

### 2.3. Biochemical analysis

These serum biochemical indexes levels (alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C) and high-

density lipoprotein-cholesterol (HDL-C)) were detected following the instructions of detection kits.

#### 2.4. Enzyme-linked immunosorbent assay (ELISA)

Colon and spleen samples were grinded into homogenate. The supernatant in each sample was obtained after centrifugation (3500 rpm, 10 min, 4 °C). BCA protein assay kit (Nanjing Jiancheng) was used to measure total protein contents. Levels of IL-1 $\beta$ , interleukin-2 (IL-2), IL-6, interleukin-10 (IL-10), interleukin-12 (IL-12), interleukin-17 (IL-17), TNF- $\alpha$ , interferon-gama (INF- $\gamma$ ), immunoglobulin A (IgA), secretory immunoglobulin A (sIgA), immunoglobulin G (IgG) and natural killer (NK) cells in colon, spleen or serum were detected following the instructions by ELISA kits and ELISA reader (Tecan, Salzburg, Austria).

#### 2.5. Histopathological analysis

The spleen was removed by laparotomy and fixed in neutral formaldehyde solution. The samples were embedded in paraffin wax and cut into 5  $\mu$ m. Then the sections were stained with hematoxylin-eosin (H&E) and observed under a light microscope (Nikon, Tokyo, Japan).

#### 2.6. Immunohistochemistry analysis

Immunohistochemistry was performed on the spleen to investigate expression changes of CD3 and CD20. The samples were embedded in paraffin after incubating in paraformaldehyde (4%) for 24 h, and then sliced into 5- $\mu$ m sections. Then samples were stained with CD3 and CD20 antibodies, respectively (overnight, 4 °C). Secondary antibody was added to the tissues for 2 h in the next day. The samples were observed by light microscope to analyze CD3 and CD20 expressions. Morphometric analysis of immunohistochemical results was performed by ImageJ software (version 6.0).

#### 2.7. Gut microbiota analysis

The cecal contents samples of each group were collected in sterilization tubes, and frozen in liquid nitrogen immediately. The total bacterial DNA of cecal contents was measured by E. Z.N.A.® Soil DNA kit (Omega Bio-tek, Norcross, GA, U.S.). DNA integrity were evaluated by 1% agarose gel electrophoresis. The purity was evaluated by NanoDrop (2000) (Thermo Scientific, Wilmington, USA). 16 S rRNA V3–V4 hypervariable regions from gut microbiota were amplified by PCR. These PCR amplicons were quantified and then sequenced on Illumina MiSeq platform (Illumina, San Diego, CA, USA). Sequenced reads and operational taxonomic units (OTUs) were bases of all the data.

#### 2.8. Feces metabolomics

50 mg sample was put into 1.5 mL centrifuge tube, and 400  $\mu$ L extracting solution was added. Then the sample was extracted by low temperature ultrasound. After centrifugation at 13,000 rpm for 15 min at 4 °C, the supernatant was removed and dried with nitrogen, and dissolved with 120  $\mu$ L mixture. Then the samples were extracted with low-temperature ultrasound, and centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatant was removed into an injection vial with internal tube for machine analysis. Quality control samples (QCs) were prepared by mixing aliquots of all the samples to make a pooled sample. QCs were injected at every 10 samples and provided a set in which repeatability could be assessed. Chromatographic separation was conducted on BEH C18 (100 mm  $\times$  2.1 mm, 1.8  $\mu$ m) with injection 10  $\mu$ L. The mobile phase was composed of (A) water (containing 0.1% formic acid) and (B) acetonitrile/Isopropanol (1/1) (containing 0.1% formic acid). Gradient parameters were as follows: 95–80% A and 5–20% B in 0–3 min, 80–5% A and 20–95% B in 3–9 min, 5% A and 95% B in 9–13 min, 5–95% A and 95–5% B in 13.0–13.1 min, and holding at 95% A and 5% B in 13.1–16

min. Flow rate 0.40 mL/min; column temperature 40 °C; mass range: 50–1000 m/z. Mass spectrometer operation parameters: ion spray voltage 5000 V (+) and 4000 V (–); spray gas 50 psi, auxiliary heating gas 50 psi, air curtain gas 30 psi; ion source heating temperature 500 °C; 20–60 V cyclic collision energy.

#### 2.9. Metabolomics data analysis

After selected and normalized, the data were matched with the public databases Human Metabolome Database (HMDB) and Metlin to obtain metabolite information. The data was imported into Majorbio (<https://cloud.majorbio.com>) to conduct principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). The selection of significant difference metabolites was according to the value of variable importance for projection (VIP) generated by OPLS-DA model, and *p* value generated by student's *t*-test. Differential metabolites (VIP >1 and *p* < 0.05) were chose to have further analysis by using software Origin (version 2021b) and Cytoscape (version 3.6.1).

#### 2.10. Correlation analysis of network and heatmap

To explore the mechanisms of SAV in normal mice, the data was visualized by multiscale network and heatmap. Pearson's rank correlation was applied to display parameters relations. Only correlations with Pearson's correlation coefficient < –0.6 or >0.6 and *p* < 0.05 (generated by Origin, version 2021b) were selected for network visualization. Network and heatmap were generated by using Origin (version 2021b) and Cytoscape (version 3.6.1).

#### 2.11. Statistical analysis

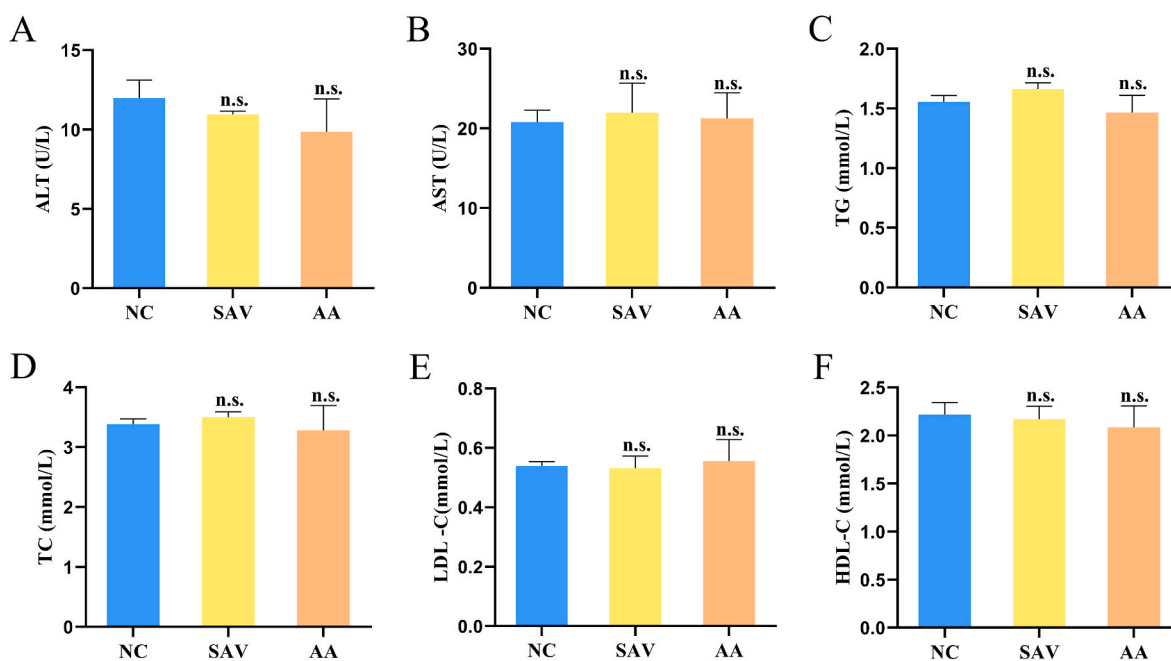
Species classification, richness diversity and microbial community functions prediction were finished on Omicsmart platform (<https://www.omicsmart.com/>). Independent *t*-test was applied to the analysis of statistical significance. All data were expressed as mean  $\pm$  standard deviation (SD) (*n* = 6–10) using GraphPad prism (version 8.0.2). All the parameters were repeated for 3 times. The levels of *p* < 0.05 represent statistically significant difference. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. NC group, n. s. indicates no significant difference.

### 3. Results and discussion

#### 3.1. Effects of SAV on host parameters in normal mice

In this study, hepatic enzymes (ALT and AST) levels in serum had no obvious differences between SAV group and NC group, and between AA group and NC group (Fig. 1A and B). In addition, there was not significant alteration among blood lipids and lipoproteins (TG, TC, LDL-C and HDL-C) after AA and SAV intake (Fig. 1C–F). Beheshti et al. reported that vinegar consumption reduced TG, TC and LDL-C levels in hyperlipidemia patients, and had no significant influence on HDL-C level (Beheshti et al., 2013). Another study reported that schisandra vinegar significantly reduced serum TG, TC and LDL-C content, and increased HDL-C content in high-fat diet rats (Yuan et al., 2020). In present study, SAV has no effects on blood lipids in normal mice. AA was the main component of vinegar, and the content of AA in SAV aged 8 years old was about 5.6% (Xia et al., 2018a). Several studies has found that AA can reduce blood lipids levels in obese organism (Kondo et al., 2009; Yamashita, 2016). However, our results showed that AA had no effects on blood lipids in normal mice. Few studies reported that the effects of vinegar and AA on normal mice. The discrepant results between our study and other reports were mainly due to different animal models.

It is well known that T helper 0 cells (Th0) can secrete IL-12, which induce Th1 cells to generate TNF- $\alpha$  and IFN- $\gamma$ . Th0 can also promote Th17 cells to produce IL-17. These factors (TNF- $\alpha$ , IFN- $\gamma$  and IL-17) further enhance the secretion of inflammatory factors (Curciarello



**Fig. 1.** Host parameters in normal mice treated by SAV and AA. Hepatic enzymes ALT (A) and AST (B) levels. Blood lipids indexes TG (C), TC (D), LDL-C (E), and HDL-C (F) levels.

et al., 2019). In our study, pro-inflammatory factors including IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  levels in serum, and IL-1 $\beta$ , IL-6, IL-12, IL-17, TNF- $\alpha$ , and IFN- $\gamma$  levels in colon were obviously decreased by SAV administration. However, AA treatment only significantly reduced the levels of IL-17 and INF- $\gamma$ , with no significant impact on other indexes in this study (Table 1). Shen et al. reported that vinegar inhibited inflammation through suppressing Th1 and Th17 responses in mice with ulcerative colitis. Inflammatory factors (INF- $\gamma$ , IL-17, IL-1 $\beta$  and TNF- $\alpha$ ) in colon were significantly inhibited after vinegar (5% v/v) and acetic acid (0.3% v/v) treatment, which showed the same protective effect (Shen et al., 2016). The inhibition of inflammation was also observed in our study, and the effect of AA on inflammatory response was less than that of SAV. Above data suggest that SAV can regulate immune response, and lower inflammatory levels in normal mice.

### 3.2. Effects of SAV on spleen immunity in normal mice

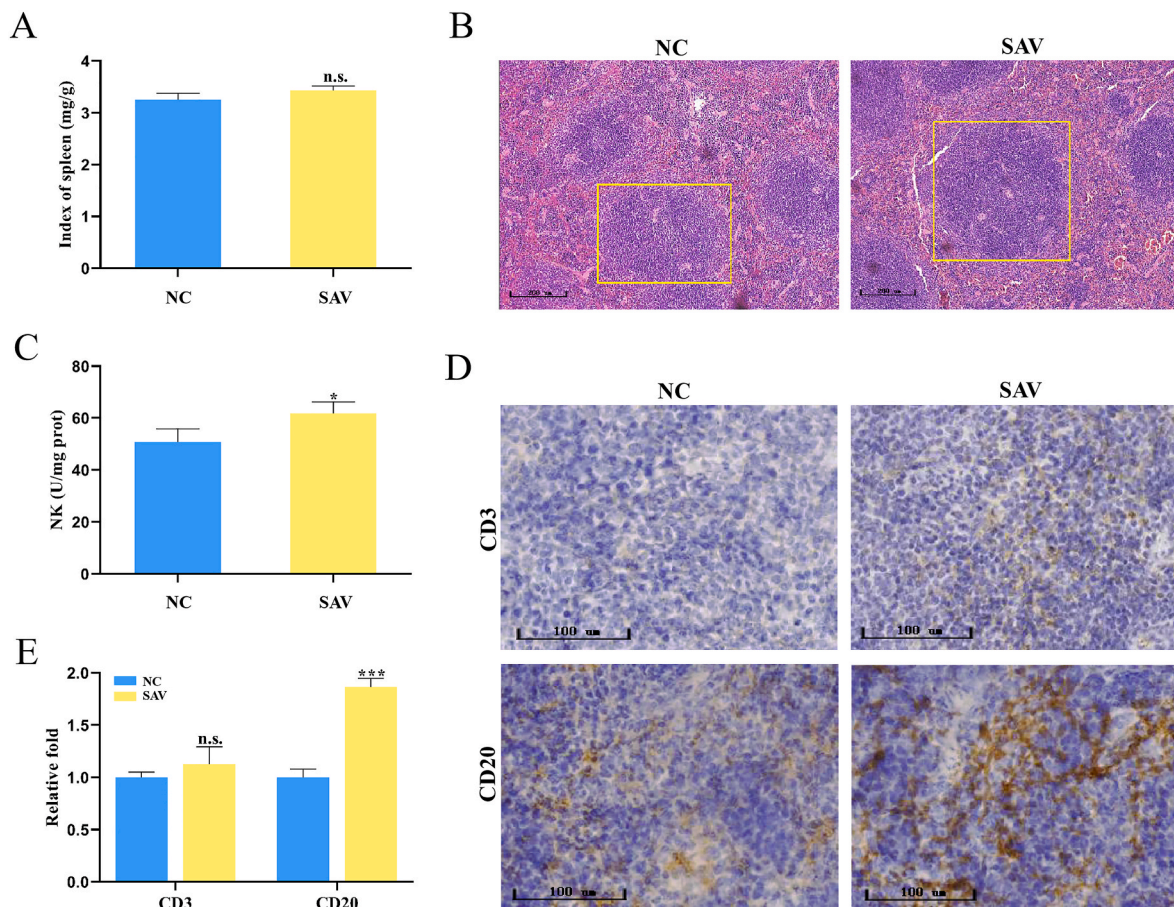
There is a central lymphoid tissue in the spleen. The increase of

**Table 1**  
Immune factors and immunoglobulins in mice.

	NC	SAV	AA
<b>Serum</b>			
IL-2 (pg/mL)	24.86 $\pm$ 2.74	23.49 $\pm$ 2.99	23.04 $\pm$ 2.08
IL-1 $\beta$ (pg/mL)	38.47 $\pm$ 2.25	33.66 $\pm$ 1.27*	34.29 $\pm$ 2.80
IL-6 (pg/mL)	60.48 $\pm$ 0.86	59.03 $\pm$ 1.14	59.86 $\pm$ 4.98
TNF- $\alpha$ (pg/mL)	204.9 $\pm$ 13.82	173.3 $\pm$ 10.69*	197.75 $\pm$ 20.64
IFN- $\gamma$ (pg/mL)	43.02 $\pm$ 4.36	32.79 $\pm$ 4.46*	39.88 $\pm$ 2.29
IL-10 (pg/mL)	200.20 $\pm$ 10.15	218.70 $\pm$ 17.69	198.34 $\pm$ 11.98
IgA (ug/mL)	270.00 $\pm$ 3.52	288.70 $\pm$ 3.23**	280.51 $\pm$ 13.04
IgG (mg/mL)	15.57 $\pm$ 0.37	16.35 $\pm$ 0.18*	15.53 $\pm$ 0.21
<b>Colon</b>			
IL-1 $\beta$ (pg/mg prot)	19.00 $\pm$ 0.69	17.60 $\pm$ 0.16*	16.82 $\pm$ 1.16
IL-6 (pg/mg prot)	32.79 $\pm$ 0.58	30.60 $\pm$ 0.53**	33.60 $\pm$ 2.42
IL-12 (pg/mg prot)	21.49 $\pm$ 0.62	19.42 $\pm$ 0.61*	20.89 $\pm$ 1.14
IL-17 (pg/mg prot)	12.03 $\pm$ 0.19	10.49 $\pm$ 0.44**	11.07 $\pm$ 0.21*
TNF- $\alpha$ (pg/mg prot)	123.10 $\pm$ 4.15	112.60 $\pm$ 3.02*	114.29 $\pm$ 6.26
IFN- $\gamma$ (pg/mg prot)	20.51 $\pm$ 0.82	17.20 $\pm$ 0.77**	16.33 $\pm$ 2.27*
IL-10 (pg/mL prot)	68.30 $\pm$ 2.75	76.13 $\pm$ 1.83*	72.75 $\pm$ 2.67
sIgA (ug/mg prot)	82.50 $\pm$ 0.18	95.43 $\pm$ 1.27***	98.62 $\pm$ 8.11

splenic nodule size is related to immune activation (Bo et al., 2016). As shown in Fig. 2A, there was no obvious difference among the spleen index between the two groups. However, the spleen nodules in the SAV group were obviously enlarged after SAV intake, indicating that SAV intake can activate lymphocytes and improve the immune responses in the spleen of normal mice (Fig. 2B). NK cells are essential for maintaining homeostasis and controlling immune response, which have a close correlation in promoting more effective Th1 cells immunity and regulating autoimmunity (Mikelez-Alonso et al., 2021). We found that the activity of NK cells was significantly increased after SAV administration (Fig. 2C), which contribute to maintain immune homeostasis in the spleen of normal mice. IL-2 is important for the proliferation of T cells and the generation of effector and memory cells (Mizui, 2019). We found that the levels of CD3 expression and IL-2 were not obvious changed in two groups (Table 1, Fig. 2D and E). Mohamad et al. reported that population of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen of normal mice were increased after coconut juice vinegar consumption for 14 days, which was associated with higher levels of serum IL-2 and IFN- $\gamma$  cytokines (Mohamad et al., 2018). In our study, there was no obvious alteration of T cells after SAV intake, this may be caused by the different raw materials, resulting in different active ingredients of the vinegars. The immunomodulatory activity of vinegar may be closely related to these bioactive compounds. The immunomodulatory activity of coconut juice vinegar may be partly due to its protein content (Mohamad et al., 2018), while grain vinegar was used in this study with low protein content. Meanwhile, the CD20 expression level were significantly added by SAV administration (Fig. 2D and E). CD20, as a B lymphocyte surface antigen, regulates the activation of B cells (Kuijpers et al., 2010). B lymphoid cells differentiate into plasmocytes, which secrete immunoglobulin and mediate immune response (LeBien and Tedder, 2008). This alteration was corresponded to the increase of IgA and IgG levels in serum (Table 1). Taken together, these results imply that SAV intake enhance immune function by improving the activities of NK cells and B lymphoid cells, and serum immunoglobulins levels.





**Fig. 2.** The SAV effects on immune function in normal mice spleen. (A) The spleen indexes. (B) Spleen histopathological was observed by H&E staining with microscopy (100× magnification). (C) The activity of NK cells was measured by ELISA kit. (D) CD3 and CD20 levels were measured by immunohistochemical staining (200× magnification). (E) The relative folds of CD3 and CD20 expression by morphometric analysis.

### 3.3. Effects of SAV on gut microbiome composition and function in normal mice

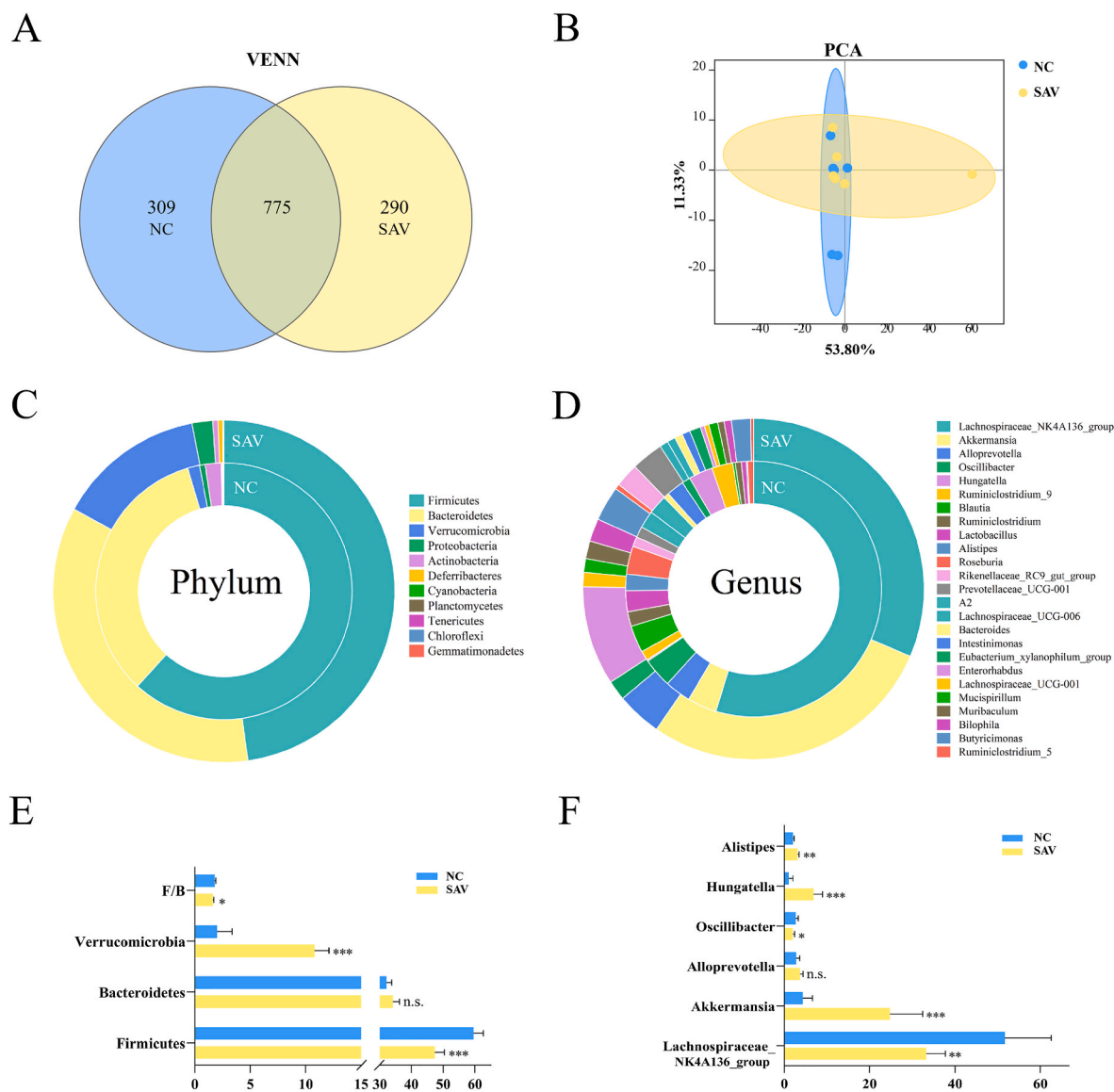
According to the sequence similarity of N97%, averages of 1163 and 1299 OTUs were identified in two groups (Table 2). A healthier individual has higher microbial richness. Good's coverage was applied to evaluate whether the sequencing method can be truly characterized gut microbiota composition (Rinninella et al., 2022). In present study, all the sequences in the two groups had nearly complete Good's coverage (Table 2). Eventhough the diversity had no clearly changes between the two groups, the richness of gut microbiota in SAV group was remarkably higher than that in NC group (Table 2), indicating the healthy status is well-being after SAV intake. In addition, Venn diagram showed that two groups had their own unique OTUs and shared OTUs (Fig. 3A), and PCA plot displayed that individuals in two groups were partly separated from their own group (Fig. 3B). These results suggest that gut microbiota profile is partly altered by SAV intake in healthy individuals.

It is well known that Firmicutes, Bacteroidetes and Verrucomicrobia

are major phyla in gut microbiota, and decrease in the abundance ratio of Firmicutes/Bacteroidetes (F/B) is associated with health benefits (Carmody et al., 2015). In our study, SAV intake significantly cut F/B ratio, which would be beneficial to health after SAV consumption (Fig. 3E, Table S1). In addition, the abundances of Verrucomicrobia *Akkermansia*, and *Alistipes* were remarkably higher after SAV intake (Fig. 3C–F, Tables S1 and S2). *Akkermansia*, a dominant genus of Verrucomicrobia, can increase the thickness of mucus and enhance the intestinal barrier function, which is negatively associated with inflammation and metabolic syndrome incidence (Dao et al., 2016). *Alistipes* have protective effects in liver fibrosis, colitis and cancer immunotherapy (Parker et al., 2020). Food and beverage products derived from fruit and vegetables are rich in polyphenols, which are closely related with health benefits. Kemperman et al. found that polyphenols extract from a red wine grape increased *Akkermansia* and *Alistipes* growth in vitro gut microbial ecosystem (Kemperman et al., 2013). In our study, SAV contains various polyphenols, and the increase of Verrucomicrobia and *Akkermansia* may promote gut barrier and decrease host inflammation. On the contrary, the abundances of Firmicutes, *Lachnospiraceae\_NK4A136\_group* and *Oscillibacter* displayed a significant decline after SAV intake (Fig. 3E and F). The genera of *Lachnospiraceae\_NK4A136\_group* and *Oscillibacter* belong to phylum of Firmicutes. Increased abundance of *Lachnospiraceae\_NK4A136\_group* and *Oscillibacter* were closely associated with ulcerative colitis, gastritis and obesity (Zhou et al., 2022). *Oscillibacter* promotes intestinal permeability, and generation of inflammatory factors (IL-6 and IL-1β) (Wu et al., 2019). Generally, our data demonstrate that dietary SAV can alter gut microbiota structure and composition, which may exhibit

**Table 2**  
Gut microbiota diversity in mice.

Groups	NC	SAV
OTUs	1163 ± 77.90	1299 ± 72.99*
Good's coverage	0.9942 ± 0.0011	0.9934 ± 0.0025
Richness	Chao1	1548.00 ± 100.20
	Ace	1628.00 ± 112.00
Diversity	Shannon	6.38 ± 0.41
	Simpson	0.96 ± 0.02
		6.56 ± 0.48
		0.97 ± 0.02



**Fig. 3.** The SAV effects on gut microbiota composition and microbial community functions prediction. (A) Venn diagram. (B) The PCA plots. (C) Composition of microbial at phylum level. (D) Relative abundances of bacterial taxa at genus level. (E) Relative abundances of Firmicutes, Bacteroidetes, Verrucomicrobia, and the ratio of F/B. (F) Relative abundances of *Lachnospiraceae\_NK4A136\_group*, *Akkermansia*, *Alloprevotella*, *Oscillibacter*, *Hungatella*, *Alistipes*.

benefit for host health.

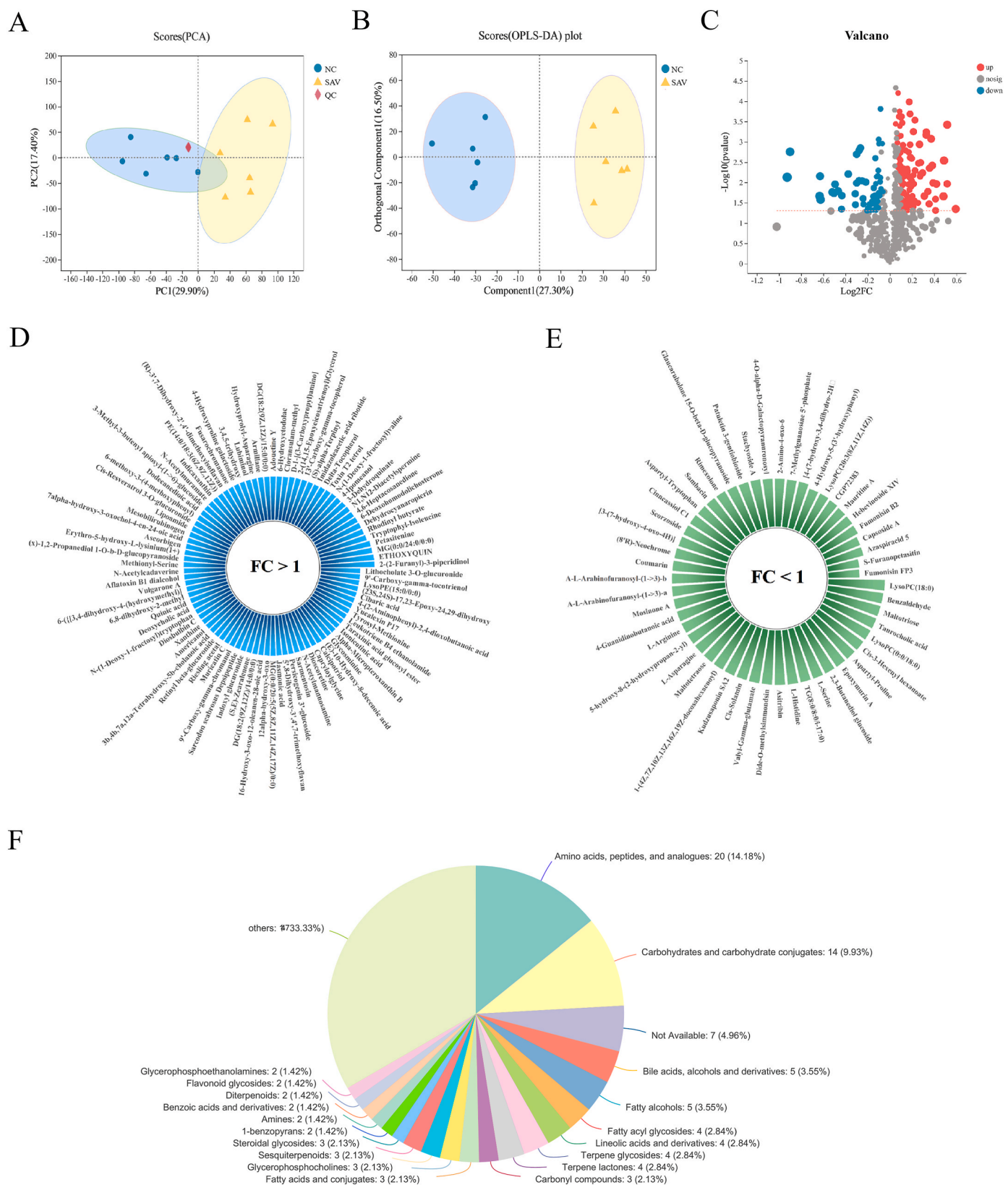
### 3.4. Effects of SAV on metabolome in the feces of normal mice

To explore the effects of SAV on metabolic profile, LC-MS was applied to detect fecal metabolome. The results showed that PCA plots displayed a clear separation of two groups in positive mode (Fig. 4A) and negative mode (Fig. S1A). In addition, OPLS-DA analysis was used to estimate metabolic fingerprints, which presented an excellent separation of two groups in positive and negative modes (Fig. 4B and Fig. S1B), indicating that the established method had good stability and repeatability. Then, VIP value and *p* value were applied to be the screening criteria for identifying differential metabolites. 141 differential metabolites between NC and SAV group were shown in volcano plot in positive and negative modes (Fig. 4C). 90 of these 141 differential metabolites were up-regulated, and 51 of them were down-regulated in the SAV-treated group compared with NC group (Fig. 4D and E). These results suggest SAV consumption also alter gut metabolome in normal mice. The classification of these metabolites was analyzed based on HMDB database (Table S3). In the classification pie-chart, the differential

metabolites between the two groups mainly included 20 amino acids, peptides, and analogues (AAPA) (14.18%), 14 carbohydrates and carbohydrate conjugates (CCC) (9.93%) and 5 bile acids, alcohols and derivatives (BAAD) (3.55%) (Fig. 4F). Gut metabolites generated by gut microbiota is important in maintaining health and disease development (Wang et al., 2019). Johnston et al. found that daily vinegar ingestion for 4 weeks significantly increased 3 amino acid metabolites, including sarcosine, piperic acid and acetylcysteine, 3 fatty acid metabolites (3-adipic acid, lauric acid and caproic acid), and 2 carbohydrate metabolites in healthy adults (Johnston et al., 2021). The alteration of amino acid metabolites was associated with mood improvement. We also found amino acids metabolites were the main metabolite alterations, which might predict the mood benefit after SAV intake.

### 3.5. Correlation between gut microbiome and metabolome after dietary vinegar

To further illuminate the relationship between gut microbiome and differential metabolome after dietary vinegar, the correlation analysis was conducted by Pearson correlation analysis. Differential metabolites



**Fig. 4.** The SAV effects on fecal metabolism in normal mice. (A) PCA plot in positive mode. (B) The OPLS-DA scores plot in positive mode. (C) Volcano plot of variables between NC group and SAV group. (D) Metabolites of significant differences were up-regulated in SAV group. Fold change (FC) > 1 means up-regulation of metabolites. (E) Metabolites of significant differences were down-regulated in SAV group. FC < 1 means down-regulation of metabolites. (F) Metabolites of significant differences between two groups were classified according to HMDB classification.



associated with gut microbiota were selected and displayed in heatmap, including 11 AAPA, 8 CCC, 3 BAAD, and others (Fig. 5). It is reported that Firmicutes can be significantly increased by using amino acids from dietary food, and promotes the occurrence of intestinal inflammatory reaction (Zeng et al., 2022; Lin et al., 2023). Tanes reported that Firmicutes play an important role in amino acid metabolites, which that may modify host health (Tanes et al., 2021).

In our study, Firmicutes and *Oscillibacter* were mainly correlated with AAPA (indicaxanthin, L-asparagine, and aspartyl-tryptophan). The genus of *Oscillibacter* belongs to phylum of Firmicutes, these bacteria were decreased after SAV consumption, and influenced amino acids metabolites and inhibited inflammatory reaction. We also found that *Lachnospiraceae\_NK4A136\_group* were closely associated with AAPA and fatty acids and conjugates. *Akkermansia* is the representative of Verrucomicrobia phylum, which consumes amino acids for protein synthesis (Sharon et al., 2019). Kordy et al. found that *Akkermansia* abundance was decreased, which induced the increase of gene expression of amino acid metabolism in the liver disease (Kordy et al., 2021). In our study, Verrucomicrobia and *Akkermansia* presented obviously association with the fecal metabolites including 6 AAPA, 6 CCC and 4 BAAD and 2 fatty acids and conjugates. Tu et al. found that a black raspberry-rich diet selectively enhanced *Akkermansia* population in normal C57BL/6 J mice, which impact aromatic amino acid metabolism and carbohydrate metabolism (Tu et al., 2018). Another study reported that *Akkermansia*

administration showed correlation with bile acids metabolism, which alleviated obesity and non-alcoholic fatty liver disease in mice (Juárez-Fernández, et al., 2021). Generally, our data indicated that SAV intake may improve host health via gut microbiome-related metabolism.

### 3.6. Multi-dimensional network and heatmap analysis of SAV

In present study, the metabolites were chosen from AAPA, CCC, BAAD, and fatty acids and conjugates with false discovery rate <0.05. As shown in Fig. 6A, 19 host indexes, 17 metabolites, and 7 taxa in gut microbiota were integrated into the network. The nodes in host indexes (green circle), gut microbiota layer (purple circle), and fecal metabolite layer (blue circle) were closely connected with each other. Then, the relationship between gut microbiota, metabolites and host indexes was presented in heatmaps (Fig. 6B). 3 differential gut microbiota (Firmicutes, Verrucomicrobia, and *Akkermansia*), 6 differential metabolites (Maltotetraose, L-Histidine, 4-O-alpha-D-Galactopyranuronosyl-D-galacturonic acid, (x)-1,2-Propanediol 1-O-b-D-glucopyranoside, Taur-ocholic acid 3-sulfate, 3 b,4 b,7a, 12a-Tetrahydroxy-5b-cholanoic acid) were more correlated with host indexes.

Emerging data proved that gut metabome such as amino acid metabolites, short-chain fatty acids and bile acids played a bridge role between gut microbiome and host physiological function (Ursell et al., 2014). L-histidine is an amino acid derived from dairy food, which can

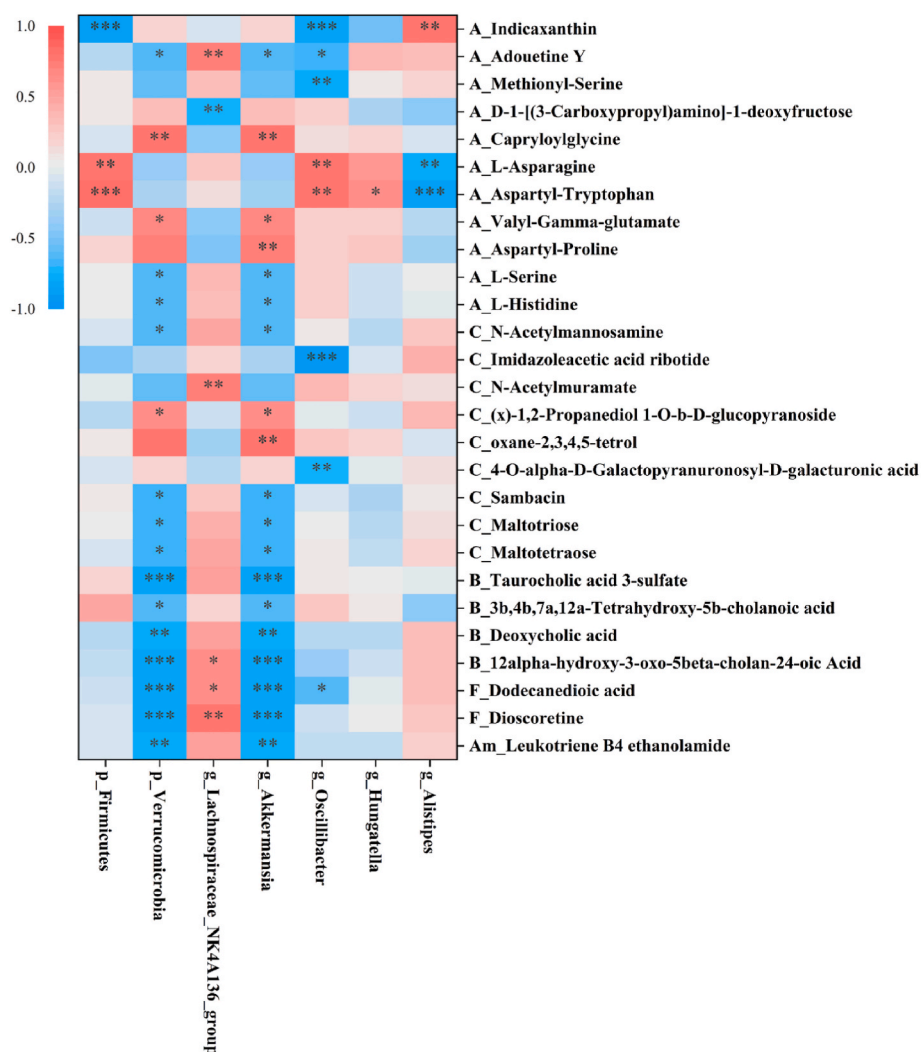
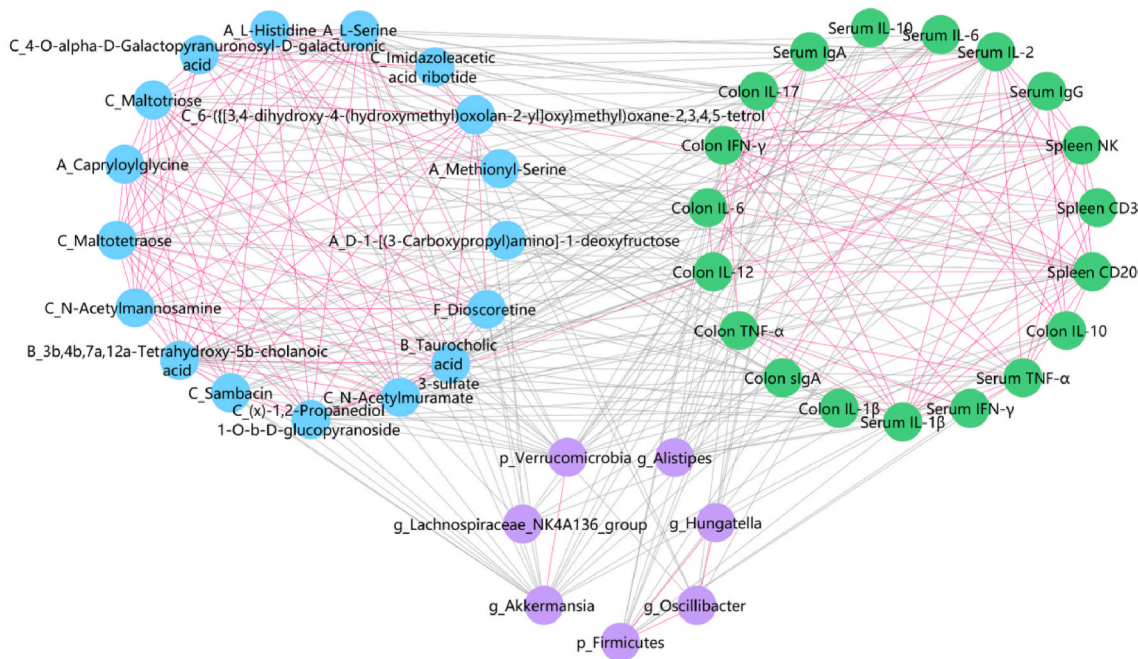


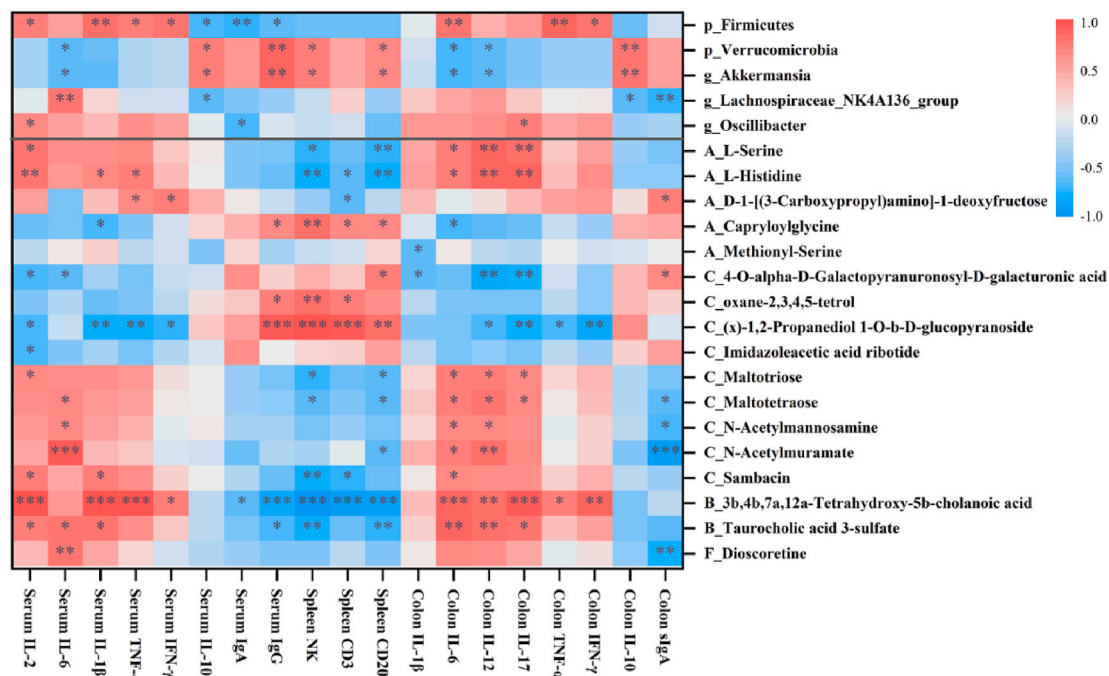
Fig. 5. Correlation analysis between differential gut microbiota and metabolites. Classification of metabolites were described at the abbreviation in metabolite name (A: AAPA, B: BAAD, C: CCC, F: fatty acids and conjugates, Am: amines).



A



B



**Fig. 6.** Correlations among gut microbiota, metabolites and indexes by Pearson correlation analysis. (A) Multi-dimensional network analysis among gut microbiota, fecal metabolites and immune factors. The edges are showed when Pearson’s correlation coefficient  $< -0.6$  or  $> 0.6$  and  $p < 0.05$ . Edges in the same classification layer are pink color, and the edges in different classification layers are black color. (B) Heatmap of gut microbiota and metabolites, and indexes. Classification of metabolites were described at the abbreviation in metabolite name (A: AAPA, B: BAAD, C: CCC, F: fatty acids and conjugates). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

be obtained through protein hydrolysis. Stražar et al. found that *Akkermansia* and *Bifidobacterium* mediated histidine metabolism, and affected cytokines (IL-6, TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ) as immunomodulatory pathways (Stražar et al., 2021). In our study, L-histidine was closely correlated with *Verrucomicrobia*, and *Akkermansia* (Fig. 5), and essentially influenced serum IL-2, IL-1 $\beta$ , and IFN- $\gamma$ . These factors as potential pathways to further modulate inflammation and immune.

Gut microbiota plays a key part in regulating host energy-balance and immune response by participating in carbohydrate metabolism (Wang et al., 2019). It has been reported *Akkermania* participated in amino acid metabolism and carbohydrate metabolism, which conferred health benefits (Tu et al., 2018). After SAV intake, we discovered carbohydrate metabolites were closely related with *Verrucomicrobia*, and *Akkermansia* (Fig. 5), and affected influenced serum IL-2, IL-6, spleen NK

cells and CD20, colon IL-12 and IL-17. These data indicated that gut bacterial especially Verrucomicrobia, and *Akkermansia* altered carbohydrate metabolism, and then involved in immune homeostasis by modulating immune cells and factors. Juárez-Fernández et al. reported that *Akkermansia* was linked to bile acids metabolism, and caused with the amelioration of liver steatosis and inflammation in mice with obesity and non-alcoholic fatty liver disease (Juárez-Fernández et al., 2021). 3 b,4 b,7a, 12a-Tetrahydroxy-5b-cholanoic acid is known as a kind of polyhydroxylated bile acids, can reduced inflammatory and fibrotic genes, and protected from cholestatic liver in mice (Ling et al., 2012). We found that bile acid metabolism was mainly affected by Verrucomicrobia, and *Akkermansia* (Fig. 5), and exhibited significantly associated with inflammatory and immune response. Collectively, the results revealed that SAV consumption caused remarkable upregulation of Verrucomicrobia, and *Akkermansia* and had an impact on the metabolisms of amino acids, carbohydrates, and bile acids, and further affected the host anti-inflammation and immunoregulation process, which exert beneficial health effects.

#### 4. Conclusion

This study illustrated the effects of SAV intake on host indexes, gut microbiome, and fecal metabolome of normal mice. SAV administration increased immunoglobulin and the splenic immunity, and inhibit serum and intestine inflammation. In addition, SAV intake made the structure of gut microbiota tend to a healthy state. SAV intake can regulated intestinal metabolites including AAPA, CCC, and BAAD. SAV was able to maintain body health involved in the interaction among physiological parameters, gut microbiota, and metabolites. Furthermore, the gut metabolism changes of amino acids, carbohydrates and bile acids significantly affected by Verrucomicrobia, and *Akkermansia*, which were association with some immunoregulation factors and inflammatory indexes. These findings provide crucial evidence for beneficial effects of SAV, and encourage SAV further applications as a functional food for human health.

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#### Ethics statement

We ensured that all the animal procedures were performed in compliance with relevant laws and the guidelines of the institutional animal ethics committee. Animal experiments were approved by the animal ethics committees of Nankai University (SYXK, 2019–0001, permission date: Jan 11, 2019).

#### CRedit authorship contribution statement

**Ting Xia:** conducted research and drafted the manuscript. **Chaoyan Kang:** was responsible for elaboration of main data and figures. **Xiao Qiang:** designed the proposal and edited the paper. **Xiaodong Zhang:** designed the proposal and edited the paper. **Shaopeng Li:** did some experiments and data analysis work. **Kai Liang:** did some data analysis work and submitted amendments to the manuscript. **Yiming Wang:** did some experiments and data analysis work. **Jianxin Wang:** submitted amendments to the manuscript. **Hui Cao:** provided some suggestions and approved the final version of manuscript. **Min Wang:** provided some suggestions and approved the final version of manuscript, All authors read and approved the final version of this manuscript.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crfs.2023.100566>.

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