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Using gut microbiota and non-targeted metabolomics techniques to study the effect of xylitol on alleviating DSS-induced inflammatory bowel disease in mice

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Abstract

Background Inflammatory bowel disease (IBD) has become a global healthcare issue, with its incidence continuing to rise, but currently there is no complete cure. Xylitol is a widely used sweetener in various foods and beverages, but there is limited research on the effects of xylitol on IBD symptoms.

Aim Study on the effect of oral xylitol in improving intestinal inflammation and damage in IBD mice, further explore the mechanism of xylitol in alleviating IBD symptoms using intestinal microbiota and non-targeted metabolomics techniques.

Methods An IBD mouse model was induced using sodium dextran sulfate (DSS). After 30 days of oral administration of xylitol, we assessed the disease activity index (DAI) scores of mice in each group. The expression levels of inflammatory factors in the colon tissues were measured using qPCR. Additionally, we examined the damage to the intestinal mucosa and tight junction structures through HE staining and immunohistochemical staining. Finally, the alterations in the gut microbiota of the mice were analyzed using 16S rDNA sequencing technology.The production of three main short-chain fatty acids (SCFAs, including acetate, propionic acid and butyric acid) in feces and the changes of serum metabolomics were measured by non-targeted metabolomics techniques.

Results The findings indicated that xylitol effectively mitigated weight loss and improved the DAI score in mice with IBD. Moreover, xylitol reduced the expressions of Caspase-1, IL-1β, and TNF-α in the colon tissue of the mice, and increased the expressions of ZO-1 and occludin in intestinal mucosal. Xylitol could enhance the variety of intestinal bacteria in IBD mice and influenced the abundance of different bacterial species. Additionally, metabolomic analysis

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revealed that oral xylitol increased the levels of three main SCFAs in the feces of IBD mice, while also impacting serum metabolites.

Conclusions Our findings suggest that xylitol can help improve IBD symptoms. Xylitol can improve the intestinal flora of IBD mice and increase the production of SCFAs to play an anti-inflammatory role and protect the mucosal tight junction barrier. These discoveries present a fresh prophylactic treatment of IBD.

Clinical trial number Not applicable.

Keywords Xylitol, Inflammatory bowel disease, Gut microbiota, Metabolomics, Inflammation, Tight junction

Background

IBD is a long-term inflammatory disorder that mainly affects the digestive system, encompassing Crohn's disease (CD) and ulcerative colitis (UC) [1, 2]. As non-developed countries and regions such as Asia, South America, and the Middle East have experienced industrialization and urbanization in the past few decades, IBD has become a worldwide ailment [3–5]. In China, there has been a notable rise in the occurrence of IBD in recent years, making it a common and frequently encountered illness [6].

The exact cause of IBD is still not fully understood and is believed to be influenced by genetic susceptibility, mucosal immunity, and the intestinal microecological environment [7, 8]. In recent years, there has been growing interest in exploring the connection between the intestinal microflora and the development of IBD [9, 10]. According to the prevailing theory, IBD is a result of an exaggerated immune response in individuals who are genetically predisposed, triggered by the presence of intestinal flora disorder. As a result, various microecological treatments have been investigated for their potential in managing IBD [11, 12].

Xylitol, a natural sugar alcohol, is commonly found in fruits and vegetables and can also be produced by the human body itself [13, 14]. It is widely recognized as a safe edible sweetener. Recent research has confirmed its health benefits, including lowering blood sugar levels and preventing dental caries [15, 16]. Xylitol has been widely used as the most important sucrose substitute in food production, including chewing gum, various beverages, and pastries. The total amount of xylitol consumed by IBD patients in their daily diet is increasing [17, 18]. A current unavoidable issue is whether xylitol has a beneficial or harmful impact on IBD patients.

With its low calorie content, only a small amount of xylitol is directly absorbed by the body, with 5% excreted [19, 20]. The majority of xylitol is digested by intestinal microorganisms, and its role in the intestinal tract should not be underestimated, though there is limited research in this area. In addition, based on the close relationship between intestinal dysbiosis and the onset and progression of IBD, exploring the role of xylitol in regulating the dysbiosis of intestinal flora in IBD patients is of great significance for the treatment and daily diet of IBD. This study induced IBD in mice through oral administration of dextran sulfate sodium (DSS), and elucidated the prophylactic treatment of xylitol on IBD in mice by analyzing its regulatory effect on intestinal flora and serum metabolomics disorders. The results of this study can provide guidance for the clinical treatment and daily diet of IBD, and provide theoretical support for the widespread use of xylitol as a sugar substitute.

Results

Xylitol improved body weight loss and DAI score deterioration in mice

When treated with DSS for 3 days, the IBD group mice showed significant soft stools. On the fifth day, mice in the IBD group tested positive for fecal occult blood. From day 7 onwards, the weight of mice in the IBD group was the lowest among the three groups. The mice in the xylitol group began to show obvious stool soft and fecal occult blood positive on the 5th day of the experiment. From day 5 of DSS induction, DAI scores were significantly higher in the IBD and xylitol groups than in the NC group, but no difference between the IBD and xylitol groups (Fig. 1).

Xylitol reduces intestinal mucosal damage and increases the expressions of tight junction proteins ZO-1 and occludin

The HE staining and immunohistochemical staining results for ZO-1 and Occludin showed no differences between the NC group and the xylitol control group, indicating that adding 5% xylitol to the drinking water had no significant effect on the integrity of the intestinal mucosal barrier in mice (Fig 2).

Compared to the NC group, DSS mice had significantly shortened colonic villi, ulcers in the mucosa and submucosa, and infiltration of neutrophils and lymphocytes. The Xylitol group mice had normal colonic villi with only mild infiltration of neutrophils and lymphocytes, while the NC group mice showed no signs of inflammation (Fig. 4A-D).

ZO-1 and Occludin proteins are mainly expressed on the surface of intestinal mucosa, within the intestinal gland lumen, and between intestinal glands (Fig. 4E-L).

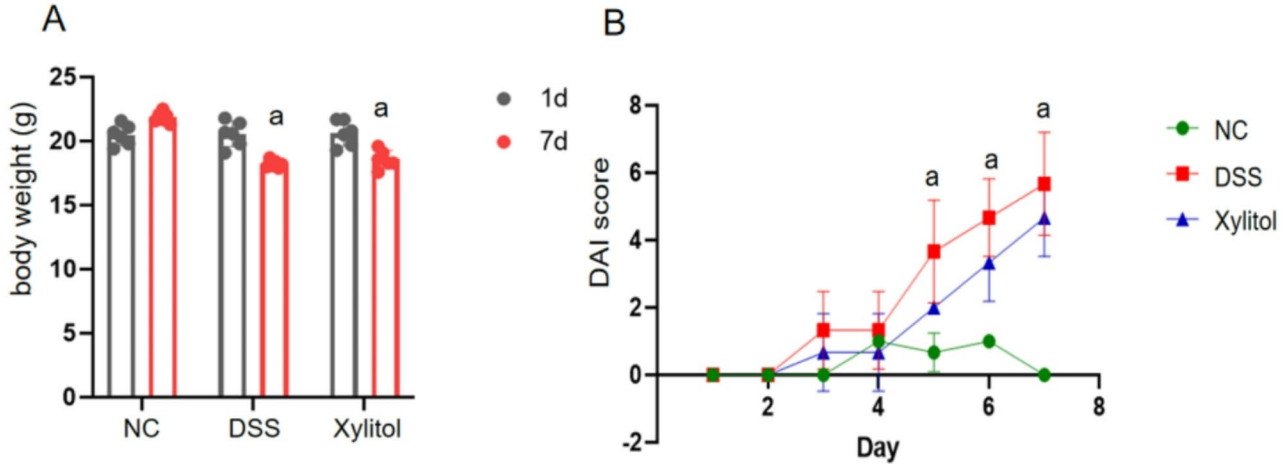

Fig. 1 Changes in mouse body weight and DAI score during the experiment. A: body weight changes; B: DAI score changes. a: compared with the NC group, $P<0.05$

Compared to the NC group, the DSS and Xylitol groups of mice showed significantly decreased expression of ZO-1 and Occludin on the surface of intestinal mucosa. Compared to the DSS group, the Xylitol group mice showed a significant increase in the expression of ZO-1 and Occludin on the surface of intestinal mucosa ($P<0.05$) (Fig. 4M, N).

Xylitol suppressed pro-inflammatory cytokine expression in the colonic tissues of mice

Compared with the NC group, the expressions of NLRP3, Caspase-1, IL-1β and TNF-α were significantly increased in the DSS and xylitol groups ($P<0.05$). Compared with the DSS group, the expressions of Caspase-1, IL-1β and TNF-α were significantly increased in the xylitol group ($P<0.05$), and there was no significant change in NLRP3 expression ($P>0.05$) (Fig. 3).

Xylitol increased the productions of three main SCFAs in the mouse colon

Compared with the NC group, the concentrations of acetic acid and butyric acid in the colon contents of the DSS group significantly decreased ($P<0.05$), and no significant change in propionate concentration ($P>0.05$). The concentrations of acetic acid and butyrate in the colon contents of the xylitol group were significantly higher than those of the NC and DSS groups ($P<0.05$), and the concentration of propionic acid was only significantly higher than that of the DSS group ($P<0.05$) (Fig. 4).

Effects of xylitol on the intestinal microbiota of IBD mice

Gut microbes participate in various host physiological activities including immune defense, nutrition, metabolism and immune regulation. In this study, we explored the effect of xylitol on the intestinal flora of IBD mice. As shown in Fig. 5A-D, there was significant difference in intestinal flora α diversity between the NC and xylitol groups ($P<0.05$), but no significant difference between other groups ($P>0.05$). In terms of β diversity, the PCA scatter plot and PCoA scatter plot showed that the NC group was significantly separated from the DSS and xylitol groups, and the xylitol group was between the NC and DSS groups, indicating that the intestinal flora of the NC group was significantly different from the DSS and xylitol groups, and xylitol could improve the intestinal flora diversity of IBD mice (Fig. 5E and F).

The mouse intestinal flora was further analyzed at the phylum and genus levels (Fig. 5G and H). At the phylum level, *Fimicutes* abundance was significantly lower in the DSS and xylitol groups compared with the NC group ($P<0.05$). At the genus level, the *Akkesmansia* and *Allobaculum* abundance was significantly higher in the DSS and xylitol group compared with the NC group. Compared with the NC group, the abundances of *Bilophila*, *Odoribacter*, *Oscillospira* and *Prevotrllaceae* were significantly decreased in DSS group ($P<0.05$). Compared with the DSS group, *Allobaculum*, *Streptococcus* abundance were significantly reduced in the Xylitol group, while *Odoribacter* abundance were significantly increased ($P<0.05$). There was no significant change in the abundance of *Gemmatimonadetes* in the colon contents of the 3 groups (Fig. 6).

Effects of xylitol on serum metabolomics in IBD mice

The various metabolites in mouse serum were analyzed by UPLC-Q-TOF-MS to explore the effect of xylitol on serum metabolites in IBD mice. The PCA plot indicated a better separation between samples from the the NC, DSS, and xylitol groups. Further using OPLS-DA analysis revealed significant separation between the NC and DSS groups, the NC and xylitol groups, the DSS and xylitol

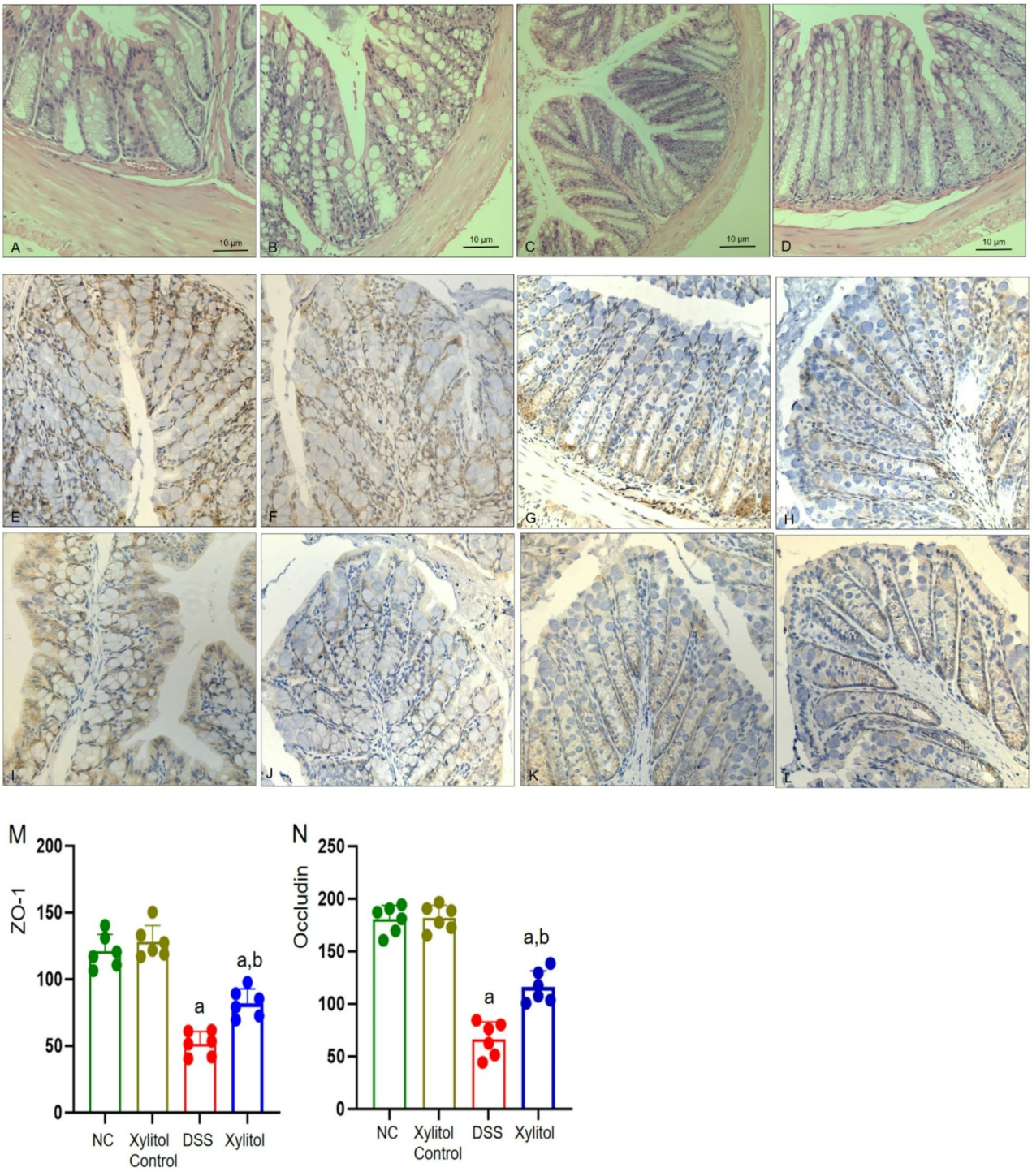

Fig. 2 The results of mouse intestinal mucosa HE staining and tight junction proteins ZO-1 and Occludin immunohistochemical staining ($n=3$). **A**: NC group HE staining; **B**: Xylitol Control group HE staining results; **C**: DSS group HE staining results; **D**: Xylitol group HE staining results; **E**: NC group ZO-1 immunohistochemical staining; **F**: Xylitol Control ZO-1 immunohistochemical staining; **G**: DSS group ZO-1 immunohistochemical staining; **H**: Xylitol group ZO-1 immunohistochemical staining; **I**: NC group Occludin immunohistochemical staining; **J**: Xylitol Control group Occludin immunohistochemical staining; **K**: DSS group Occludin immunohistochemical staining; **L**: Xylitol group Occludin immunohistochemical staining; **M**: ZO-1 protein expression level; **N**: Occludin protein expression level

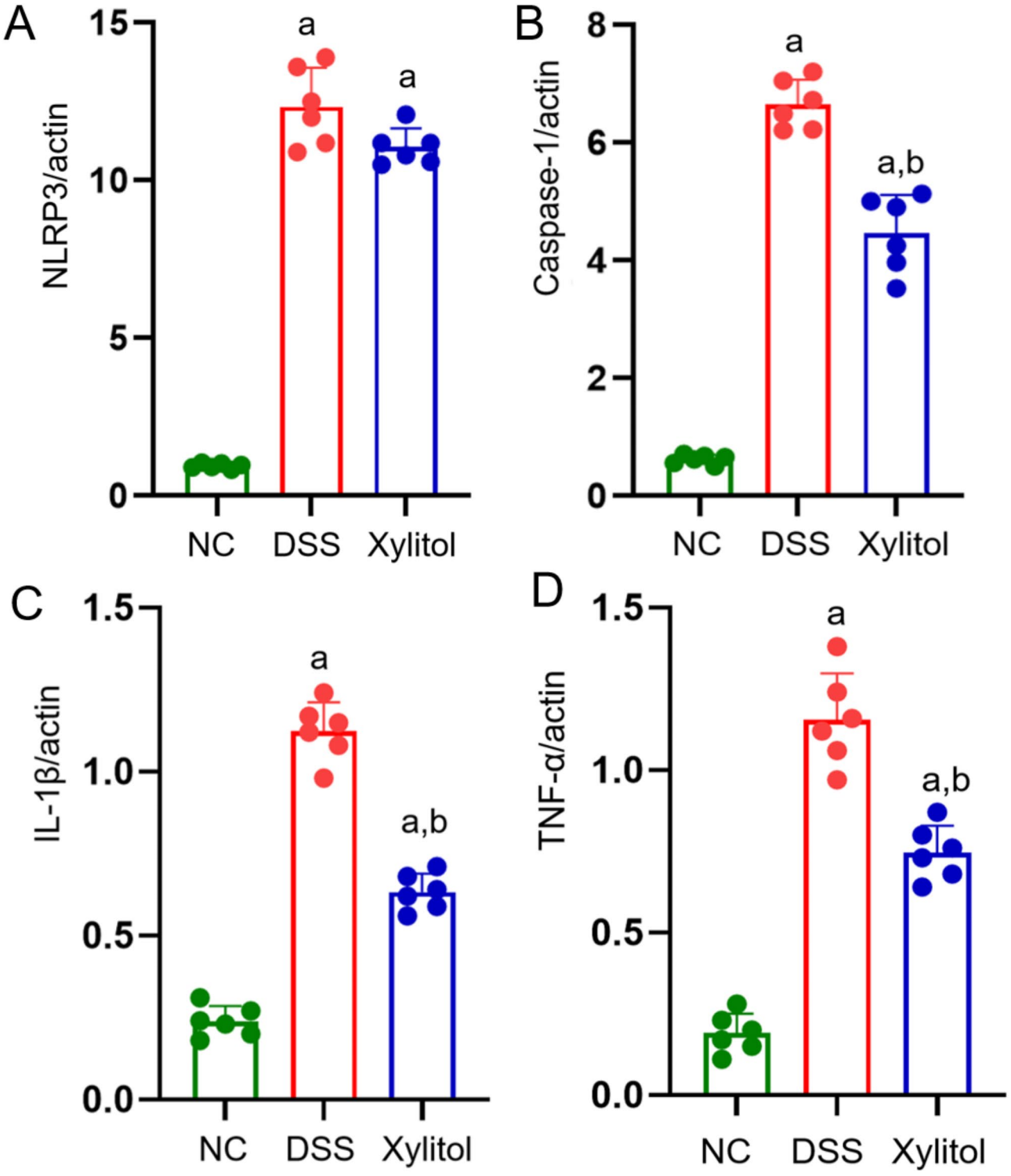

Fig. 3 The mRNA expressions of proinflammatory cytokines in mouse colon tissue ($n=6$). **A**: NLRP3; **B**: Caspase-1; C: IL-1β; D: TNF-α. **a**: compared with the NC group, $P<0.05$; **b**: compared with the DSS group, $P<0.05$

groups. The differential metabolites were screened using the rule of VIP > 1 and $P < 0.05$ (Fig. 7).

From the PCA scatter plot, the samples of the NC and DSS groups could not be completely distinguished, and the samples of the DSS and xylitol groups could be significantly distinguished without outlier samples. For further OPLS-DA analysis, the abscissa was the predicted principal component score and the ordinate was the

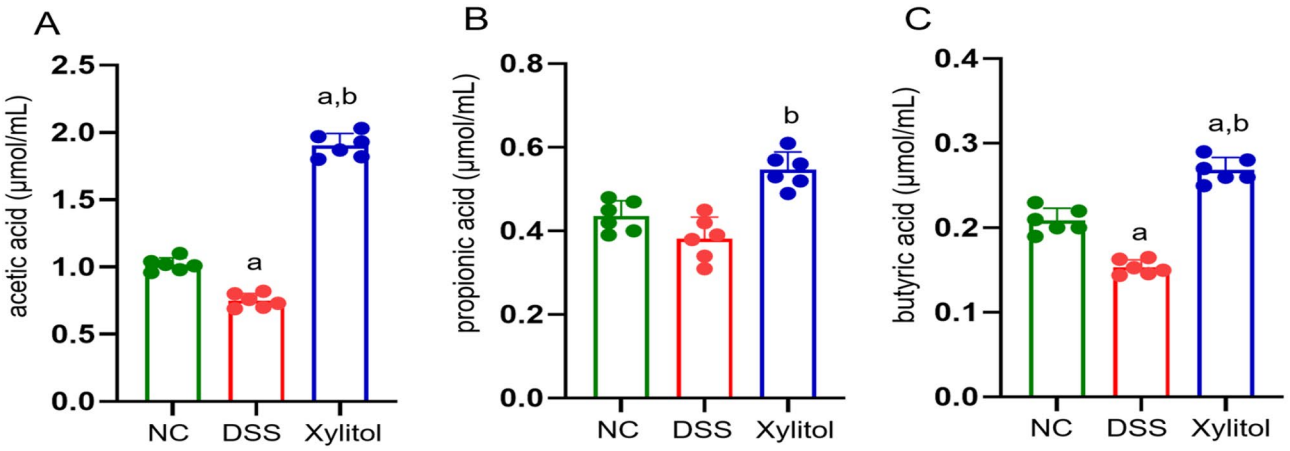

Fig. 4 Concentrations of acetic acid, propionic acid, and butyric acid in the mouse colonic contents ($n=6$). **A**: acetic concentration; **B**: propionic acid concentration; **C**: butyric acid concentration. **a**: compared with the NC group, $P<0.05$; **b**: compared with the DSS group, $P<0.05$

orthogonal principal component score. As shown in the Fig. 7, the DSS group coud be separated from the xylitol group (ESI^- $R^2X=0.507$, $R^2Y=0.316$, $Q^2=0.76$). The above results showed that the metabolites between the NC and DSS groups, and the DSS and xylitol groups were significantly different.

23 differential metabolites were screened from the NC and DSS groups (Table 1). Compared with the NC group, the main metabolites up-regulated in the DSS group were 5b-Cholestane-3a, 7a, 12a, 23 S, 25-pentol, 3a, 7b, 21-Trihydroxy-5b-cholanoic acid, Myristoylglycine and 19-Norandrosterone; the main metabolites down-regulated were Enkephalin L and Mevalonic acid.

24 different metabolites were screened from serum of the DSS group and xylitol groups (Table 2). Compared with the DSS group, Oleoyl glycine, Nervonic acid, Oleic acid and Pantothenic acid were the main metabolites up-regulated in serum of the xylitol group, while Palmitoyl glucuronide and Norepinephrine sulfate were also down-regulated. The clustering heat map could reflect the increase or decrease of the relative content of different metabolites in each group. Compared with the NC group, the relative content of differential metabolites in the DSS group was significantly changed, and the intervention of xylitol could reverse this change.

The metabolic pathways of the differential metabolites between the NC and DSS groups, the DSS and xylitol group were analyzed, and a total of 6 related metabolic pathways were found, namely D-glutamine and D-glutamate metabolism, nitrogen metabolism, purine metabolism, tryptophan metabolism, primary bile acid biosynthesis and biotin metabolism. Xylitol can reverse the metabolic pathways that are disrupted by DSS (Fig. 8).

Disscussion

With the increasing use of sugar substitutes in food and beverages, there is a growing concern for the safety of these alternatives [21, 22]. Xylitol, derived from agricultural crops like corncob and bagasse, is a commonly used sugar substitute. It is a natural sweetener and has been internationally recognized as a safe food substance [23, 24]. Unlike other sugar substitutes, xylitol not only provides sweetness but also has nutritional value. It is the sweetest among all polyols and its sweetness is comparable to sucrose. Additionally, xylitol is an intermediate of human sugar metabolism [25, 26]. Even in a healthy individual who does not consume xylitol-containing foods, there is still a small amount of xylitol present in their blood (0.03 to 0.06 mg per 100 mL), and the human liver can produce 5 g to 15 g of xylitol per day [27]. Since xylitol is a normal part of sugar metabolism in the body and does not require insulin for its metabolic process, it can be safely consumed by diabetic patients without causing a rise in blood sugar levels.

The existing research on xylitol suggests that it has numerous benefits in the food and pharmaceutical industries as a sugar substitute, as well as in preventing dental and physical diseases [28]. Xylitol has been found to have a significant anti-plaque effect on teeth and can help reduce gum inflammation [27]. It also acts as a preventive agent for dental caries by inhibiting the growth of harmful bacteria [29]. Moreover, xylitol has shown potential in reducing constipation, diabetes, obesity, and other physical ailments, indicating its positive impact on the digestive and immune systems in humans [30, 31]. However, there is currently no research on the effects of xylitol on inflammatory bowel disease.

Additionally, further investigation revealed that xylitol significantly inhibited the expression of Caspase-1, IL-1β, and TNF-α in the colon tissue of IBD mice, indicating its ability to reduce inflammatory damage to the

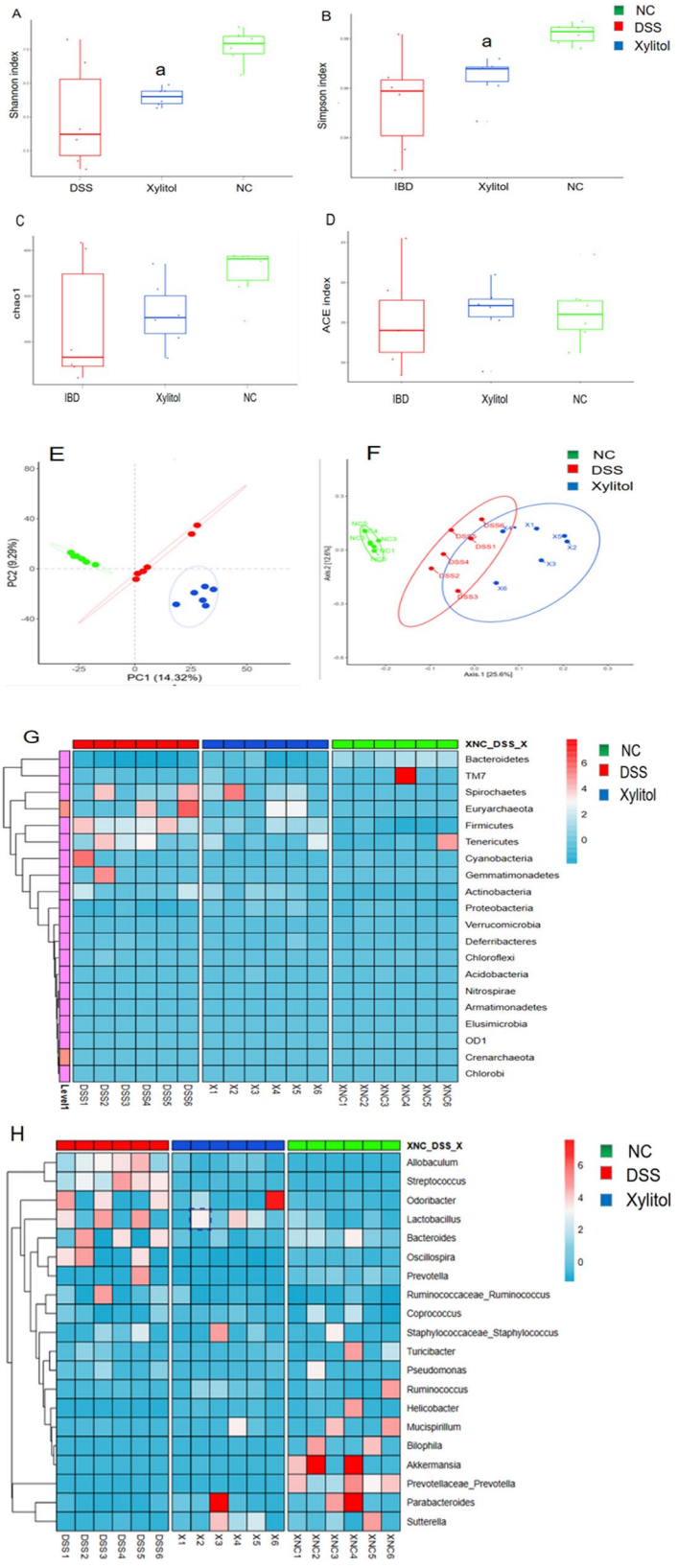

Fig. 5 Results of intestinal flora detection in mice ($n=6$). **A**: α diversity (Shannon index). **B**: α diversity (Simpson index). **C**: α diversity (Chao1 index). **D**: α diversity (ACE index). **E**: PCA scatter plot. **F**: PCoA scatter plot. **G**: phylum level clustering heat map; H: genus level clustering heat map. a: compared with the NC group, $P<0.05$

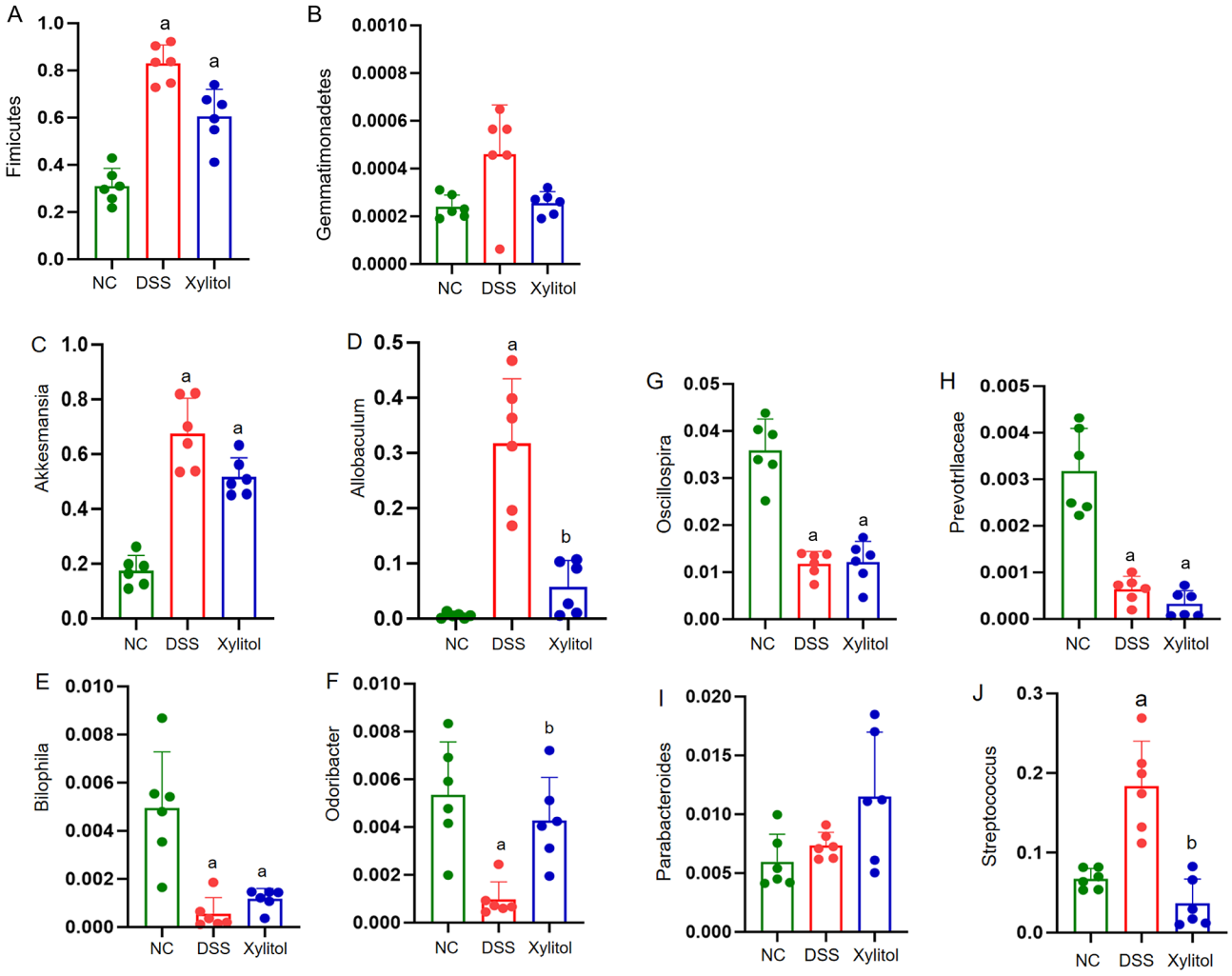

Fig. 6 Changes in the abundance of important bacteria in the intestinal flora of mice at phylum and genus levels ($n=6$). **A**: *Fimicutes*; **B**: *Gemmatimonadetes*; **C**: *Akkesmansia*; **D**: *Allobaculum*; **E**: *Bilophila*; **F**: *Odoribacter*; **G**: *Oscillospira*; **H**: *Prevotrllaceae*; **I**: *Parabacteroides*; **J**: *Streptococcus*. **a**: compared with the NC group, $P<0.05$; **b**: compared with the DSS group, $P<0.05$

colon mucosa. Furthermore, LC-MS/MS analysis of mouse feces showed that oral xylitol increased the production of acetic acid, propionic acid, and butyric acid. This finding aligns with previous research reporting the prebiotic function of xylitol [27, 29, 31]. Short-chain fatty acids (SCFAs), including acetic acid, propionic acid, and butyric acid, are organic acids produced by anaerobic bacteria during the fermentation of dietary fiber and resistant starch in the colon [32]. The characteristic of IBD is the alteration in the composition of the intestinal microbiota, with a significant decrease in the number of bacteria producing SCFAs, including bacteria from the *Firmicutes* phylum such as *Lachnospiraceae* and *Ruminococcaceae* [33, 34]. Numerous studies have highlighted the diverse biological functions of SCFAs, including anti-infection, anti-tumor, and anti-inflammatory properties, with particular emphasis on their anti-inflammatory effects [35, 36]. Based on these findings, we propose that xylitol may undergo anaerobic fermentation in the colon, leading to the production of SCFAs.

The anaerobic bacteria *Bacteroides* in the colon primarily produce acetic acid and propionic acid [37, 38], while *Firmicutes* mainly produce butyric acid [39]. These SCFAs have the ability to regulate intestinal mucosal immunity by influencing the function of various immune cells [40, 41]. In cases of acute inflammation, the balance of intestinal immune homeostasis is disrupted, leading to the release of pro-inflammatory cytokines such as IL-6, IL-8, and IL-17. These cytokines further contribute to intestinal inflammation. However, SCFAs can counteract this inflammatory response by inhibiting the production of pro-inflammatory cytokines like TNF-α, IL-6, and IL-12. This inhibition is achieved through the HDACs pathway or activation of the GPCR41/43 pathway [42]. Additionally, SCFAs can down-regulate the expression of inducible nitric oxide synthase (iNOS), vascular cell

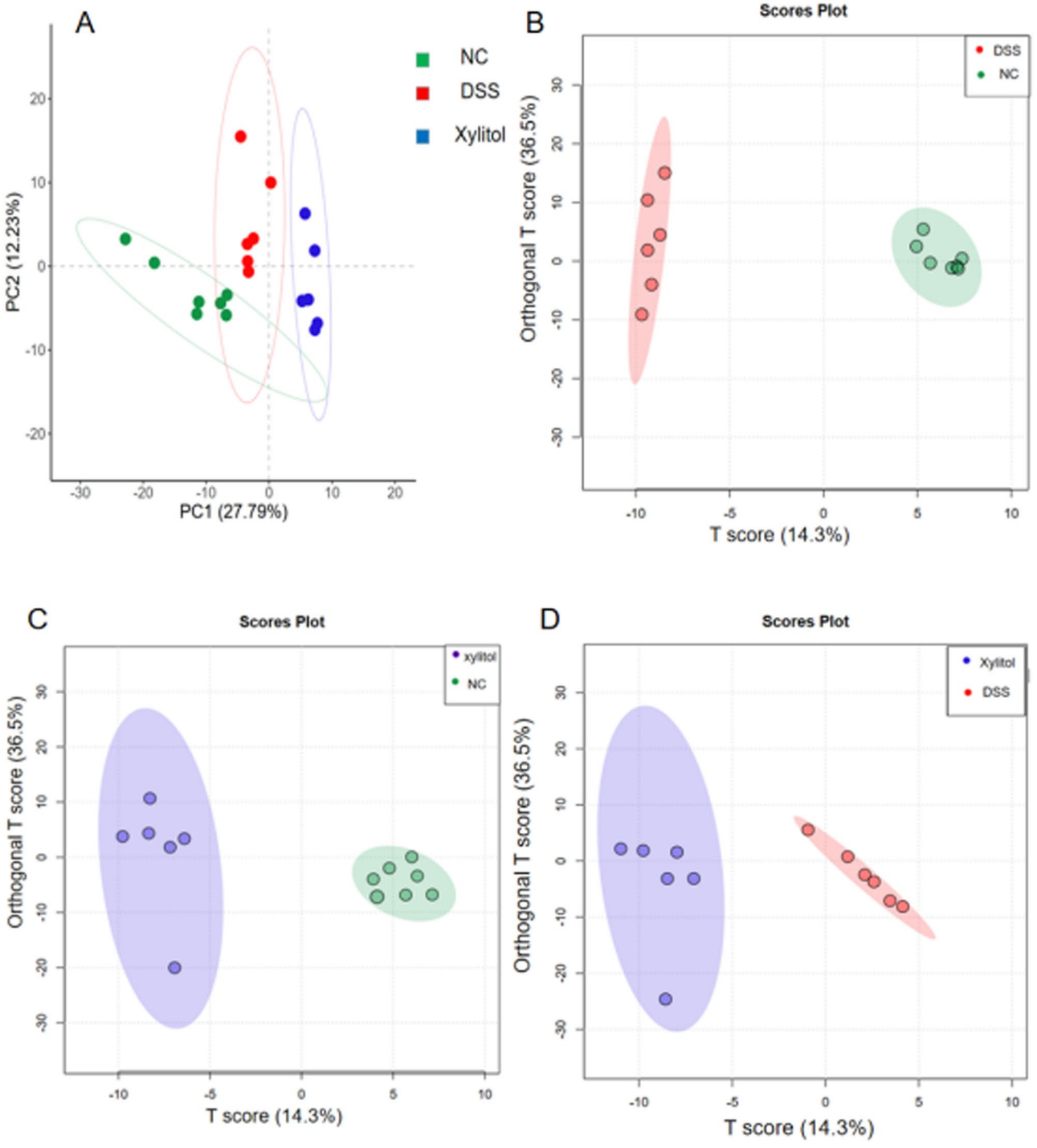

Fig. 7 Mouse serum metabolites PCA plot and OPLS-DA plot. **A**: PCA plot; **B**: OPLS-DA plot of the NC and DSS groups; **C**: OPLS-DA plot of the NC and xylitol groups; **D**: OPLS-DA plot of the DSS and xylitol groups

adhesion molecule-1 (VCAM-1), intracellular adhesion molecules (ICAM-1), and other chemokines. This down-regulation promotes the secretion of anti-inflammatory factors such as IL-10 and IL-4 by intestinal immune cells [43]. Going forward, our study will further explore the production of these three SCFAs in the colon of mice with varying levels of xylitol intake.

The results of 16S rDNA sequencing of mouse colon contents in this study also support that oral xylitol can increase SCFAs production in colon and inhibit intestinal inflammation. The abundance of *TM7* and *Fimicutes*

Table 1 Differential metabolites between the NC and DSS groups

No.	Compounds	HMDB	m/z	Retention time (min)	Vs. NC group
1	PEP-16:0/18:1(11Z))	HMDB0011341	722.5090606	12.1105	↓
2	Methyldopa	HMDB0011754	192.0657325	4.13065	↑
3	Demethylcalabaxanthone	HMDB0030656	755.2858414	4.151266667	↑
4	C.I. Acid Violet 49	HMDB0033385	710.2330728	4.165016667	↑
5	Enkephalin L	HMDB0001045	554.2607109	14.3992	↓
6	N-Acetyl-L-tyrosine	HMDB0000866	204.0657023	5.065266667	↓
7	Mercaptopurine	HMDB0015167	172.990482	3.368	↓
8	Mevalonic acid	HMDB0000227	129.0552581	3.842166667	↓
9	Dimethyl (1R*,2 S*,3 S*)-2-carboxy-3-(3,4-dihydroxyphenyl)-2,3-dihydro-5,6-dihydroxy-1 H-indene-1-acetate	HMDB0031955	433.1135707	3.904	↓
10	3-Methoxy-4-Hydroxyphenylglycol sulfate	HMDB0003332	245.0116782	3.972716667	↓
11	2,2-Dihydroperoxypropane	HMDB0034260	89.02389832	1.078983333	↑
12	5b-Cholestane-3a,7a,12a,23 S,25-pentol	HMDB0000483	473.324753	6.9344	↑
13	3a,7b,21-Trihydroxy-5b-cholanoic acid	HMDB0013192	405.2633616	6.989383333	↑
14	Myristoylglycine	HMDB0013250	569.454148	11.38156667	↑
15	pitavastatin	HMDB0041991	402.1503117	9.594833333	↓
16	Bopindolol	HMDB0015696	361.1917875	11.00361667	↑
17	19-Norandrosterone	HMDB0002697	275.2008334	275.2008334	↑
18	Muricatacin	HMDB0034110	265.2166945	11.1618	↑
19	Aspidospermatine	HMDB0030358	337.1916966	11.18241667	↑
20	Cucurbic acid	HMDB0029388	233.1155678	11.18928333	↑
21	Momordol	HMDB0029804	421.3314339	11.20303333	↑
22	Tetronasin	HMDB0033494	622.3486739	10.00036667	↑
23	Carbon disulfide	HMDB0036574	196.8864849	15.40238333	↓

in the colon of mice increased after oral administration of xylitol. Both *TM7* and *Fimicutes* are associated with the production of SCFAs in the gut [44]. After oral administration of fructooligosaccharide, *TM7* abundance and SCFAs production increased significantly [45]. When mice were fed a diet rich in resistant starch, the abundance of *Fimucutes* in the colon was also increased, as was the concentration of SCFAs [46]. *Allobaculum* is a specific marker bacteria in the stool of patients with inflammatory bowel disease, and its abundance was significantly increased in the stool of patients with IBD. In this study, we also found that the abundance of *Allobaculum* was significantly increased in the colon contents of mice induced by DSS [47, 48]. *Streptococcus* in the gut is a pathogenic bacterium that is generally considered to be one of the causes of IBD [49]. Previous studies have shown that the severity of ulcerative enteritis is proportional to the level of *Streptococcus* related antibodies in patients. It is worth noting that *Streptococcus* is also a colonizing bacterium in the human oral pharynx, and *Streptococcus* in the intestine is probably derived from ectopic colonization by oral pharyngeal bacteria [50]. Therefore, the role of oral *Streptococcus* and pharyngeal bacteria in the pathogenesis of IBD is a problem worthy of further study.

Further non-targeted metabolomics studies showed that xylitol can regulate D-glutamine and D-glutamic acid metabolism, primary bile acid biosynthesis metabolism, tryptophan metabolism, nitrogen metabolism, purine metabolism and biotin metabolism in IBD mice, which may improve the symptoms of IBD mice through the above metabolic pathways. Among the screened differential metabolites, the role of Oleoyl glycine in the inflammatory response was unclear, but its serum concentration was reduced in IBD patients [51, 52]. In this study, the serum Oleoyl glycine level in the xylitol group was significantly up-regulated compared with the DSS group, which may be related to the treatment of IBD by xylitol, but the exact mechanism remains to be further studied.

Nervonic acid is a lipid metabolite that is significantly reduced in vivo during acute and chronic inflammation [53], possibly due to the inhibition of lipid metabolism during inflammation and the conversion to the synthesis of the pro-inflammatory metabolite arachidonic acid, which results in a substrate deficiency leading to Nervonic acid synthesis [54]. We hypothesize that the high level of inflammation in IBD patients leads to the obstruction of lipid metabolism in the liver, forcing the synthesis of many beneficial fatty acids (such as Nervonic

Table 2 Differential metabolites between the DSS and xylitol groups

No	Compounds	HMDB	m/z	Retention time (min)	Vs. DSS group
1	Oleoyl glycine	HMDB0013631	723.5	11.86	↑
2	Ethyl oleate	HMDB0034451	309.32	12.52	↑
3	Vignatic acid A	HMDB0033599	552.27	11.67	↑
4	Mercaptopurine	HMDB0015167	172.99	3.72	↓
5	Schidigeragenin B	HMDB0035506	449.26	11.67	↑
6	Tsugarioside B	HMDB0035509	615.42	12.09	↑
7	Amphotericin B	HMDB0014819	944.46	11.60	↑
8	Acoric acid	HMDB0000001	249.14	10.74	↑
9	Fluorescein	HMDB0014831	313.05	1.09	↓
10	Methyldopa	HMDB0011754	192.06	4.13	↓
11	Bifonazole	HMDB0015583	619.28	10.34	↑
12	Nervonic acid	HMDB0002368	365.34	11.27	↑
13	Ambolic acid	HMDB0035713	515.37	12.61	↑
14	Delphinidin	HMDB0003074	337.01	1.09	↓
15	Oleic acid	HMDB0000207	281.24	14.99	↑
16	Santene hydrate	HMDB0000064	279.23	15.00	↑
17	Antimony	HMDB0004118	165.9	15.29	↑
18	Palmitoyl glucuronide	HMDB0010331	439.26	5.81	↓
19	Pristanoylglycine	HMDB0013303	709.6	12.90	↑
20	Molybdate	HMDB0012260	184.87	1.66	↑
21	Blasticidin S	HMDB0030452	843.39	4.55	↓
22	Chloroform	HMDB0029596	280.82	1.09	↑
23	Norepinephrine sulfate	HMDB00002062	294.02	1.83	↑
24	Pantothenic acid	HMDB0000210	240.08	4.57	↑

acid) to shift to the synthesis of pro-inflammatory lipids. There is a high level of inflammation in obese patients, where multiple pro-inflammatory lipid metabolites (such as Omega-6 fatty acids) accumulate and further aggravate the inflammatory response [55]. In addition, we also noted a significant increase in Oleic acid levels in IBD mice after oral xylitol. Studies have shown that appropriate concentrations of Oleic acid can significantly inhibit various chronic inflammatory reactions [56, 57], which may be related to xylitol improving IBD symptoms.

Palmitoyl glucuronide is a specific metabolite in the serum of patients with ulcerative colitis, and its concentration is proportional to the severity of ulcerative colitis and is related to the level of chronic inflammation in patients [58, 59]. In this study, we found that the Palmitoyl glucuronide level in IBD mice was significantly reduced after oral administration of xylitol, which may also be related to the ability of xylitol to inhibit chronic inflammatory response, but the exact mechanism remains to be further studied.

In this study, we noted an interesting phenomenon of significantly increased pantothenic acid levels in IBD mice that took xylitol orally. Studies have shown that the deficiency of dietary nutrients such as pantothenic acid and zinc can aggravate IBD symptoms, and the symptoms can improve after supplementation of nutrients such as pantothenic acid [60]. Other studies have found that nutrition-related amino acid metabolism, nitrogen metabolism, purine metabolism, etc. can affect the occurrence and development of IBD [61], which also provides a new idea for the study of the pathogenesis and treatment of IBD. Our xylitol study also provides a pathway for the prevention and prophylactic treatment of IBD.

Conclusion

Xylitol is a common dietary additive, and the intake of xylitol is increasing in modern society. IBD is a kind of chronic disease closely related to the living habits of modern society, and there is no cure. Our results show that oral xylitol does not aggravate IBD symptoms, but inhibits colon inflammation and protected the And protect the intestinal mucosa tight junction structure. Xylitol, similar to prebiotics, can increase the production of three main SCFAs in the colon and exert its anti-inflammatory ability. Intestinal flora sequencing analysis showed that xylitol could improve intestinal flora diversity and increase the abundance of SCFAs-producing bacteria in IBD mice. Metabolomics analysis showed that xylitol can regulate the disordered D-glutamine and D-glutamate metabolism, primary bile acid biosynthesis metabolism, tryptophan metabolism, nitrogen metabolism, purine metabolism and biotin metabolism in IBD mice, which may be related to the improvement of IBD symptoms, but the exact mechanism remains to be further studied. However, the conclusion that existing xylitol can improve IBD is still based on research results from animal models. The next stage requires conducting corresponding clinical studies to confirm the impact of xylitol on IBD.

Methods

Experimental animals and groups

The male ICR mice (22 g ± 4 g) were purchased from Nanjing Wukong Biotechnology Co., Ltd. (Nanjing, China) and were maintained in the Experimental Animal Center of Jiangsu University. Relevant studies were conducted after approval by the Ethics Committee and the Experimental Animal Management and Use Committee of Jiangsu University (protocol code UJSIACUC-AP-2022032016). IBD in mice was induced by oral dextran sodium sulfate (DSS, CAS: 9011-18-1, MP Biomdicals, California, USA), as follows. The xylitol was purchased from Sigma-Aldrich (>99%, CAS: 87-99-0, USA). ICR mice were randomly divided into 3 groups, including normal control group (NC group), DSS group and xylitol group, with 6 mice in each group. Mice in the NC group

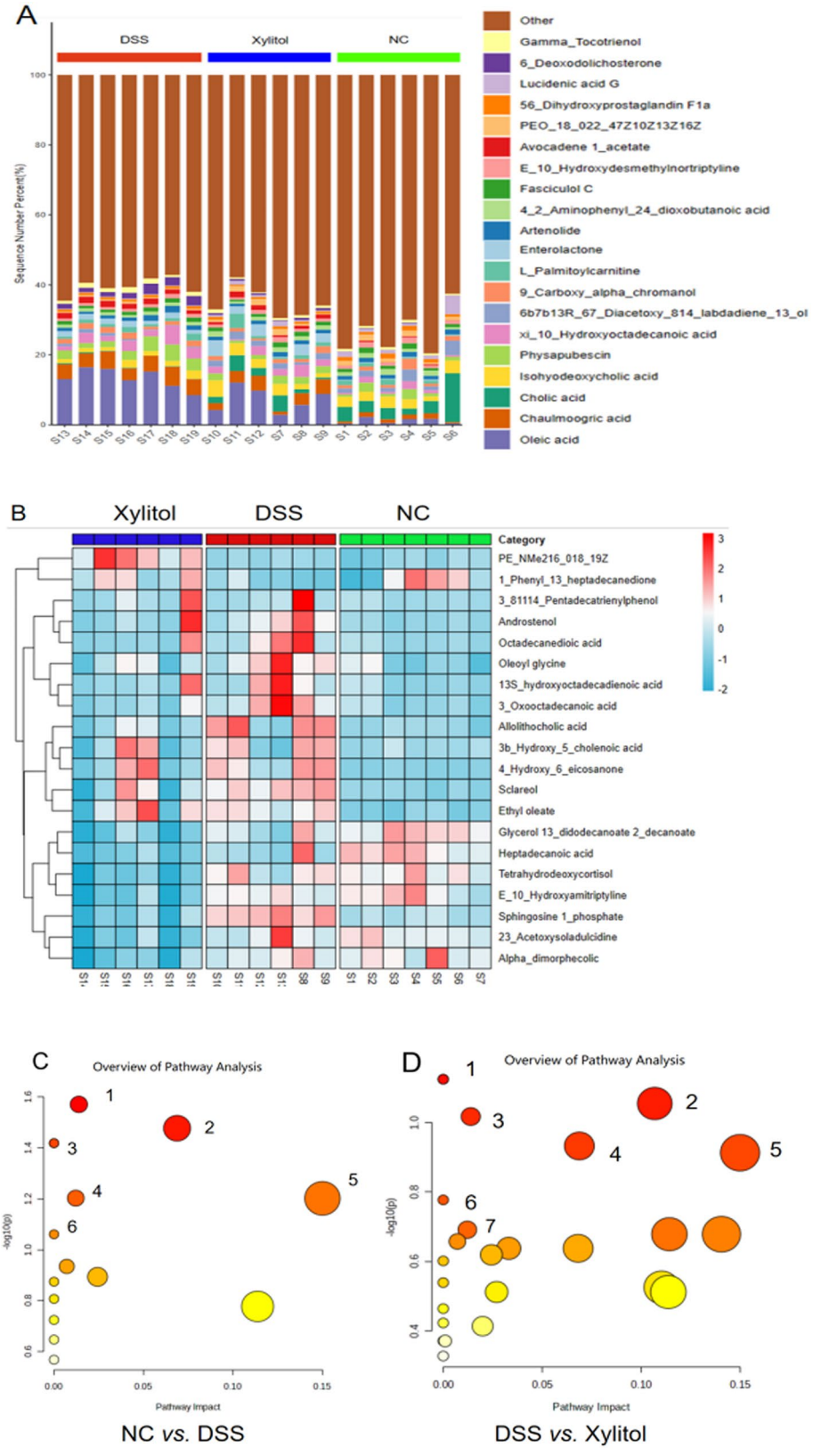

Fig. 8 (See legend on next page.)

(See figure on previous page.)

Fig. 8 Differential metabolites and related metabolic pathways in mouse serum. **A**: Map of differential metabolite percentage accumulation in mouse serum; **B**: Clustering heat map of differential metabolites in mouse serum; **C**: Analysis of metabolic pathways of differential metabolites in the NC and DSS groups mouse serum. 1: Tryptophan metabolism. 2: Primary bile acid biosynthesis. 3: D-glutamine and D-glutamate metabolism. 4: Ammonia metabolism. 5: Purine metabolism. 6: Sphingolipid metabolism. **D**: Analysis of metabolic pathways of differential metabolites in the DSS and xylitol groups mouse serum. 1: D-glutamine and D-glutamate metabolism. 2: Ammonia metabolism. 3: Pyrimidine metabolism. 4: Tryptophan metabolism. 5: Primary bile acid biosynthesis. 6: Purine metabolism. 7: Glycolysis/gluconeogenesis

were given sterilized purified water, the IBD and xylitol groups mice were given the water with 3% DSS (*v*/*v*) and drinking for 1 week to induce IBD. Referring to the xylitol experimental concentration of other scholars, mice in the xylitol group were given the water with 5% xylitol (v/v) and drinking for one month before inducing IBD [25, 62]. To further verify that oral 5% xylitol has no effect on the intestinal mucosal function of mice, we established a xylitol control group. Mice in this group continuously drank distilled water containing 5% xylitol for 30 days, and the integrity of their intestinal mucosal barrier was assessed using HE staining and immunohistochemical staining. From the 5th week, the xylitol group mice were supplemented with 3% DSS to induce IBD. Mouse weight changes, fecal traits and occult blood in feces were recorded from when the DSS was added to drinking water. At the end of the experiment, the mice were euthanized by intraperitoneal injection of Urethan (Sigma-Aldrich, St. Louis, MO, USA, 700 mg/kg), the serum and colon contents of mice were collected, and part of the colon tissue was collected for RNA extraction and qPCR analysis.

Mouse intestinal tissue HE and immunohistochemical staining

Mouse colon tissue was fixed with 4% paraformaldehyde and dehydrated with alcohol, followed by embedding in paraffin. After dewaxing with xylene, the tissue was stained with hematoxylin and eosin (HE staining). After staining, the samples were sealed with neutral gum and observed under a microscope to observe the morphological changes in the mouse colon tissue. Immunohistochemistry was used to evaluate the expression levels of the tight junction proteins including ZO-1 and Occludin in mouse colon tissue. The antibodies of ZO-1 and Occludin were provided by Boster Biological Technology Co.,Ltd (Wuhan, China), and the antibodies were diluted 1:200 for use. After dewaxing the mouse colon tissue slices, the activity of peroxidase in the samples was quenched with 3% hydrogen peroxide. After antigen retrieval, the samples were blocked with goat serum for 30 min and then used for ZO-1 and Occludin protein immunohistochemical staining. Using Image-Pro Plus image analysis software to measure the average integrated optical density of positive products in the intestinal mucosa of each group of mice, the expression levels of ZO-1 and Occludin proteins are reflected by the size of the average integrated optical density value.

QPCR assay

The total RNA kit (RC101, Vazyme, Nanjing, China) was used to extract RNA from colon tissue. Then, 1 μg of the total RNA was transcribed into cDNA using the HiScript III 1st Strand cDNA Synthesis Kit (R323-01, Vazyme, Nanjing, China). The expression levels of the target genes were determined using the AceQ Universal SYBR qPCR Master Mix Kit (Q111-02, Vazyme, Nanjing, China). The primers were synthesized by Suzhou GENEWIZ Company, and their sequences are as follows (Table 3): The qPCR reaction system consisted of 10 μL SYBR Master Mix, 0.4 μL of upstream and downstream primers (10 μmol/L), and 1 μL of cDNA. The reaction was predenatured at 95 °C for 20 s, denatured at 95 °C for 5 s, annealed at 60 °C for 20 s, and extended at 72 °C for 30 s for 40 cycles. Each sample was repeated three times, and β – actin gene was used as the internal reference to calculate the relative expressions of each target proinflammatory cytokines gene in mouse colon tissue by formula $2^{-\Delta\Delta Ct}$.

IBD model severity score

The IBD severity, including weight loss, hematochezia and stool, was assessed by DAI scoring criteria as shown in Table 4. Mouse fecal occult blood was tested by the O-linked toluidine method kit (ML095013, Shanghai yuanye Bio-Technology Co., Ltd, Shanghai, China) according to the instructions of the kit. Fresh feces from mice were collected daily by adding 2 drops of reagent A (O-toluidine solution), then 2 drops of reagent B (hydrogen peroxide solution) to observe the results immediately. Dark blue in 10 s (+ +) is strong, blue green (+), no blue in 3 min is negative (-).

Analysis of the mouse intestinal microbiota

At the end of the experiment, mouse colon contents were collected and flash-frozen in liquid nitrogen and then transported to Wekemo Tech Group Co., Ltd. (Shenzhen, China) for detection and analysis. Using the fecal genomic DNA extraction kit (TIANGEN, Beijing, China), DNA was extracted from the contents of the mouse colon according to the steps outlined in the manual. The samples were then subjected to PCR amplification and sequencing analysis. The primers 341 F

Table 3 The qPCR primers sequences for mice

Genes	Primer sequences(5′→3′)
Mouse_acin	F: CATCACTGCCACCCAGAAGACTG R: ATGCCAGTGAGCTTCCCGTTCAG
Mouse_IL-1β	F: CCTGTCCTGCGTGTTGAAAGA R: GGGAACTGGGCAGACTCAAA
Mouse_TNF-α	F: AATGGCGTGGAGCTGAGA R: TGGCAGAGAGGAGGTTGAC
Mouse_NLRP3	F: AACAGCCACCTCACTTCCAG R: CCAACCACAATCTCCGAATG
Mouse_Caspase-1	F: GCACAAGACCTCTGACAGCA R: TTGGGCAGTTCTTGGTATTC

Table 4 DAI scoring criteria of mouse IBD model

Enteritis symptoms	Severity of symptoms	Score
Weight loss (%)	No significant weight loss	0
	1–5	1
	5–10	2
	10–15	3
	>15	4
Occult blood or blood in the stool	Occult blood tests (-)	0
	Occult blood tests (+)	2
	Bloody stool	4
Fecal character	Normal	0
	Loose and soft feces	2
	Severe diarrhea	4

(CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) were used to amplify the V3-V4 region of the 16S rRNA gene from the samples. High-quality sequences obtained after amplification were filtered through quality control to remove sequences that did not meet the criteria. The resulting samples were analyzed for OUT clustering, species classification, and differential microbial community analysis. Experimental data from were analyzed on the free online platform of Wekemo Bioincloud (https://www.bioincloud.tech/).

Metabolomics analysis of mouse serum

Blood was collected at room temperature for 30 min and centrifuged at 7 100×g for 10 min before separating the upper serum for metabolomics analysis. In metabolomics analysis, 200 μL of serum was mixed with 600 μL of methanol and centrifuged at 11 300 ×g for 15 min at 4 ℃, drawing 300 μL of supernatant from each sample for UPLC-Q/TOF-MS assay (Agilent 6538 Q-TOF-MS, USA), and see the supplement for details. Metabolomic analysis was performed by Wekemo Tech Group Co., Ltd. (Shenzhen, China). The data were analyzed on the free online platform of Wekemo Bioincloud (https://www.bioincloud.tech/).

The acetic acid, propionic acid, and butyric acid detection in mice serum were performed at Health Testing Center, Zhenjiang Center for Disease Control and Prevention (Zhenjiang, China) using liquid chromatography – mass spectrometry (LC – MS/MS) following He's method [63], and see the supplement for details.

Statistical analysis and metabolomics data analysis

Data analysis was performed using the SPSS 20.0 statistical software. Measurement data are expressed as mean ± standard deviation. One-way ANOVA for comparison between multiple groups, and the least significant difference (LSD) method was used for the comparison between the two sample groups. In $P<0.05$ was considered statistically significant.

For the metabolomics data analysis, the chromatograms of serum samples were extracted using Markerview 2.1 software and the peak area was normalized. The normalized data were subjected to principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The Differential metabolites were screened by the variable important in projection (VIP) values of the OPLS-DA model variables, and the endogenous differential metabolites were screened by VIP > 1 and $P<0.05$ rules. Pathway analysis of the discovered differential metabolites was performed by MetaboAnalyst 3.0, further combined with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/kegg/kegg1.html) and Human Metabolome Database (HMDB) database (https://hmdb.ca/) to resolve the biological significance of the differential metabolites, and finally identified potential biomarkers and related metabolic pathways closely related to IBD mouse induced by DSS and treated with xylitol.

Abbreviations

IBD	Inflammatory bowel disease
DAI	Disease activity index
DSS	Sodium dextran sulfate
SCFAs	Short-chain fatty acids
CD	Crohn's disease
UC	Ulcerative colitis
NLRP3	Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein 3
IL-1β	Interleukin-1β
TNF-α	Tumor necrosis factor-α
ZO-1	Zona Occludens-1
PCA	Principal component analysis
PCoA	Principal coordinate analysis
OPLS-DA	Orthogonal partial least squares-discriminant analysis
VIP	Variable importance in the projection

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Author contributions

P M: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. W S: Writing – review & editing, Methodology, Investigation, Data curation. C S: Writing – review & editing, Methodology, Investigation, Data curation. J T: Writing – original draft, Methodology. X D: Writing – original draft, Validation, Methodology. J H: Writing – review & editing, Resources, Methodology. A A: Writing –

review & editing, Writing – original draft, Methodology, Formal analysis.M C: Methodology, Investigation, Data curation. L Z: Writing – original draft, Validation, Methodology, Funding acquisition, Data curation, Conceptualization. L W: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. P W Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request. The 16S rRNA sequence raw data of this study have been deposited in Genome Sequence Archive (PRJCA036199).

Declarations

Ethics approval and consent to participate
Relevant studies were conducted after approval by the Ethics Committee and the Experimental Animal Management and Use Committee of Jiangsu University (protocol code UJSIACUC-AP-2022032016). All methods were carried out following relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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