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Attenuation of arsenic-induced gut injury in mice by sulfated glycosaminoglycan from swim bladder *via* regulation of gut microbiota, metabolites, and intestinal barrier integrity

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ABSTRACT

Arsenic, a known environmental carcinogen, disrupts intestinal homeostasis, posing a significant threat to human health. Mitigating its toxic effects is crucial, and this study explores the potential of swim bladder sulfated glycosaminoglycan (SBSG) in achieving this. Our previous *in vitro* studies have shown that SBSG to ameliorate arsenic-induced damage in intestinal epithelial cells, but its *in vivo* effects remain elusive. The current investigation demonstrates that SBSG exhibits a beneficial prebiotic action *in vivo*, regulating gut microbiota, metabolites, and intestinal barrier function to counter arsenic's adverse effects. Specifically, SBSG regulates microbiota composition, suppressing pathogenic species like *Alistipes* and *Candidatus_Saccharimonas* while promoting beneficial ones such as *Ruminococcus* and *Akkermansia*. In the colon, SBSG fermentation enhances the production of short-chain fatty acids (SCFAs), leading to the upregulation of *GPR43*, *GPR109A*, and *Olfr78* receptors. Additionally, SBSG strengthens the intestinal barrier by increasing the expression of Claudin-1, Occludin, and ZO-1, and enhances mucin gene expression (*MUC-1* and *MUC-2*) to address chemical barrier disruptions. Immunologically, SBSG modulates the RORγt/Foxp3 pathway and the TLR4/MyD88/NF-κB signaling cascade, regulating the immune barrier. These findings suggest that SBSG could be a promising prebiotic candidate for maintaining intestinal health and may serve as a dietary supplement or adjunct in heavy metal detoxification therapies.

1. Introduction

Arsenic is a metal-like element that is often studied as a heavy metal, and it is listed as an IA environmental carcinogen by the International Agency for Research on Cancer[1]. Arsenic can enter the human body through drinking water, feeding, and inhalation[2]. In recent years, several studies have shown that arsenic exposure damaged the intestinal barrier, leading to an increase in the abundance of Bacteroidetes and Proteobacteria, and a decrease in the abundance of Firmicutes[3-4]. It promoted the enrichment of harmful microbiota and reduced the abundance of beneficial microbiota, which disrupted the structure of gut microbiota and would further adversely affect host

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metabolism and immune function[5]. Gut microbiota dysbiosis which is a pathological microbiota combination state, is a manifestation of intestinal injury caused by arsenic[6]. It is associated with various human diseases and has different dysbiosis degrees[7]. Mild dysbiosis does not cause changes in the body. Moderate dysbiosis may develop into severe gastrointestinal diseases. Severe dysbiosis can affect each organ and disturb the balance of the whole body[8]. There are many factors inducing dysbiosis, among which heavy metals are one of the most harmful factors[9]. Heavy metal exposure has adverse effects on the flora while the flora also exerts abilities to reduce heavy metal toxicity.

At present, the emerging mainstream regulation forms of attenuating heavy metal-induced gut injury are mainly divided into 2 types, one is the supplement of probiotics, and the other is the intake of prebiotics. The detoxification effects of probiotics on heavy metals have been widely studied, from traditional *Lactobacillus* to *Propionibacterium*, *Enterococcus*, *Bifidobacterium*, *Bacillus*, etc.[10-11]. In contrast, since prebiotics were proposed to alleviate the toxicity caused by heavy metals, there has been a lack of research on them[12]. It may be since although there is a certain definition of prebiotics, additional evaluation and regulations are needed to determine[13]. Furthermore, its metabolism in the body also has a certain complexity. Therefore, the establishment of a mechanism for the detoxification effects of prebiotics has certain potential significance for this field. It is found that most of the potential prebiotics are indigestible carbohydrates. The mechanism of prebiotics is that it is used by gut microbiota when they reach the lower digestive tract, producing beneficial metabolites, regulating flora, activating related signaling pathways, and a series of effects.

As a macromolecular acidic polysaccharide, glycosaminoglycan (GAG) is often considered to be unable to be absorbed and utilized in previous studies. In recent years, it has been proposed that certain degrading enzymes in the gut microbiota can decompose and utilize GAG, and it has a beneficial regulatory effect on the structure of the intestinal flora, which promotes the research on GAG as a new prebiotic[14]. Many marine organisms are rich in GAG. In promoting the high-value utilization of the swim bladder, one of the by-products of fish processing, our research group extracted a kind of GAG with a structure similar to chondroitin sulfate A (CSA) and showed that it could alleviate the damage of intestinal epithelial cells caused by arsenic exposure[15-16]. Based on this research basis and the nature of chondroitin sulfate's regulatory effect on the gut microbiota[17], this study further explored GAG regulating intestinal-related functional characteristics and sought targets to alleviate heavy metal-induced gut injury. Therefore, a short-term arsenic exposure mouse model was established and from the changes in gut microbiota structure, beneficial metabolites short-chain fatty acid (SCFA) content, SCFA-related receptors, and the expressions of intestinal barrier-related gene and protein, the regulatory mechanism of swim bladder sulfated glycosaminoglycan (SBSG) in mice with heavy metal induced-gut injury *in vivo* was studied.

2. Materials and methods

2.1 Materials and reagents

Sodium arsenite was obtained from Sigma (MO, USA). The RNA Extraction Kits, Reverse Transcription Kits and ChamQ Universal SYBR qPCR Master Mix were purchased from Nanjing Vazyme Biotech Co., Ltd. (Nanjing, China). All primers used in RT-qPCR were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and sequences of primers were shown in Table S1. Antibodies against claudin-1, occludin and ZO-1 were purchased from Proteintech Group, Inc. (Chicago, USA). Antibodies against TLR4 and MyD88 were provided by Wanleibio Co., Ltd. (Shenyang, China). Antibodies against were supplied by Cell Signaling Technology (MA, USA). Antibodies against β-tubulin, IKβα, p-IKβα, NF-κB p65 and p-NF-κB p65 were purchased from Cell Signaling Technology (MA, USA). All other reagents were analytical grade.

2.2 Preparation of swim bladder sulfate glycosaminoglycan

Swim bladder sulfated glycosaminoglycan (SBSG) of *Aristichthys nobilis* was preserved in the laboratory. The crushed swim bladder was enzymatic hydrolyzed for 24 h. It was separated and purified by anion exchange resin (Amberlite™ FPA98Cl resin), eluted with gradient sodium chloride (0.0, 0.3, 0.9, and 1.1 mol/L), and then under alcohol precipitation and freeze-drying to obtain different components. The SBSG used in this study is a higher-purity component eluted with 1.1 mol/L sodium chloride, which is composed of repeated disaccharide units of →4GlcUA β1→3GalNAc(4S)β1→, and it is a chondroitin sulfate A analog. The more detailed preparation method can refer to our previous research[15-16].

2.3 Animal experiments

Specific pathogen-free (SPF) male C57BL/6J mice (aged 7 weeks) weighing 18–20 g were purchased from Zhuhai Bestest Biotechnology Co., Ltd. (Zhuhai, Guangdong, China). Mice were housed in an environment with relative humidity 44%–70%, temperature 22–24 °C, and 12 h light-dark cycle and accessible to diet and water freely. All experimental procedures were approved by the Animal Management Committee and the Animal Ethics Committee of the Experimental Animal Center, Guangdong Ocean University (No. GDOU-LAE-2023-007), and handled following the National Research Council's Guide for the Care and Use of Laboratory Animals.

In the control group (C), normal saline was administered twice a day. The model group (M) was given normal saline and 5 mg/kg sodium arsenite solution once a day. The SBSG low-dose intervention group (GL), SBSG medium-dose intervention group (GM), SBSG high-dose intervention group (GH), and prebiotic inulin group (I) were intragastrically administered with the corresponding concentration of SBSG solution (50, 100 and 200 mg/kg) and 100 mg/kg inulin solution respectively. After 38 days, fresh feces of mice were collected for 16S rDNA gene sequencing. After fasting for 12 h, the mice were sacrificed with a collection of cecal feces, colon feces and colon samples at –80 °C for further analysis.

2.4 16S rDNA gene sequencing

The fresh feces of each group of mice were collected one day before the end of the experiment and stored in a sampling tube. The genomic DNA was extracted from feces using a nucleic acid extraction kit (NO. GHFDE100, Hangzhou Guhe Information Technology Co., Ltd., Hangzhou, China). Qualified DNA samples were applied to amplification of 16S rDNA V4 region using the universal primers 515F (GTGCCAGCM GCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT). After purification and quantification of the PCR products, the library was

established and merged into the Illumina NovaSeq sequencing platform for double-end sequencing (Paired-End) to obtain the original sequence. By splicing and filtering Reads, ASVs clustering was carried out under specific similarities. The clustering results were annotated and analyzed by QIIME2 (2020.6.0) based on silva138 database (https://www.arb-silva.de) to reveal the species composition of the sample. Subsequent sequence data analysis was performed using QIIME2 and R package (v3.2.0).

2.5 Determination of SCFAs

The sample extraction was referred to the method of Chang et al.[18], with a little change. A total of 50 mg of cecal and colonic feces of mice in each group were accurately weighed and immediately added to 50 μL of pre-cooled 15% phosphoric acid solution and 900 μL of ethyl acetate. After grinding and vortex extraction, the supernatant was obtained by centrifugation at 12 000 r/min for 10 min at 4 ℃. The supernatant was filtered by 0.22 μm organic filter membrane and tested on the triple quadrupole gas chromatography-mass spectrometry (GC-MS-TQ8050NX). The chromatographic conditions were as follows: chromatographic column GC (30 m × 0.25 mm, 0.25 μm, InertCap® Pure-WAX, Shimadzu Co., Tokyo, Japan), split injection of 1 μL at a ratio of 15:1. The temperature of the transmission line and ion source were 250, 240 and 230 ℃, respectively.

2.6 Reverse transcription qPCR analysis of genes in mouse colon

Total RNA from colon samples was extracted using an RNA extraction kit. The qualified RNA samples were reversely transcribed using a reverse transcription kit to obtain cDNA. According to the requirements of the PCR fluorescence quantitative kit, the corresponding reagents were added and used in the Bio-Rad CFX real-time PCR system for real-time quantitative PCR. In this experiment, *GAPDH* was used as an internal control gene, and $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expressions of target genes.

2.7 Determination of colonic protein expression

The colon tissue was added to the RIPA lysis buffer to obtain protein supernatant. After the protein concentration of each sample was modulated to the same, 5× SDS loading buffer was added and kept at 100 ℃ for 10 min. The protein was separated by SDS-PAGE gel, and then the separated protein was transferred to the nitrocellulose membrane. Then membranes were blocked for 1 h at room temperature in blocking buffer and then incubated with the following primary antibodies: β-tubulin (1∶1 000), claudin-1 (1∶1 000), occludin (1∶1 000), ZO-1 (1∶1 000), TLR4 (1∶1 000), MyD88 (1∶500), Ikβα (1∶1 000), p-Ikβα (1∶1 000), p65 (1∶1 000), and p-p65 (1∶1 000) overnight at 4 ℃. After several washes in washed buffer, membranes were incubated with goat anti-rabbit secondary antibodies (1:3 000) for 1 h at room temperature. Protein bands were detected by ECL enhanced kit and the signals were recorded by a chemiluminescence gel imaging system and were analyzed by ImageJ software (1.52p). β-Tubulin was chosen as the reference protein, and the relative abundance of protein expression in the colon was expressed as the abundance of targeted protein/reference protein.

2.8 Statistical analysis

The data are presented as means ± standard deviation (SD), and analyzed using one-way ANOVA combined with Duncan's test by SPSS26.0 software (IBM Co., Armonk, NY, USA). Significant levels were set as P-values < 0.05. In the analysis of intestinal flora, the α-diversity index and the β-diversity were analyzed by QIIME2 software, the species difference of microbial community was evaluated by the R software package (v.3.2.0), and the microbial function was predicted based on PICRUSt.

3. Results

3.1 Regulatory effect of swim bladder sulfated glycosaminoglycan on gut microbiota in arsenic-induced mice

Based on 16S rDNA high-throughput sequencing, the regulatory effect of SBSG on gut microbiota in arsenic-exposed mice was evaluated. In this study, the rarefaction curve showed that the number of ASVs didn't increase with the increase of sequencing depth, which meant the sequencing depth was sufficient to reflect the species information in the sample (Fig.S1), so the data were credible for subsequent analysis.

The α-diversity was used to analyze the richness, diversity, and evenness of microbial communities. As shown in Table 1, there was no significant difference in Chao1 and ACE indexes between the groups, and the Shannon and Simpson indexes were changed significantly with the increase of SBSG dose compared with the M group. The Shannon and Simpson indexes of the GL group were slightly up-regulated, and GM and GH groups were significantly down-regulated. These results indicated that SBSG had little effect on the community richness, however exerted the effect of enriching dominant bacteria in arsenic-exposure mice.

Table 1
α-Diversity indexes of all groups.

Groups	Chao1	ACE	Shannon	Simpson
C	900.89 ± 46.84^{a}	895.04 ± 43.24^{a}	6.59 ± 0.10^{a}	0.97 ± 0.00^{ab}
M	903.60 ± 40.42^{a}	924.85 ± 47.19^{a}	6.45 ± 0.16^{ab}	0.97 ± 0.01^{a}
GL	914.01 ± 69.67^{a}	902.48 ± 74.62^{a}	6.58 ± 0.18^{a}	0.98 ± 0.00^{a}
GM	863.83 ± 43.19^{a}	852.88 ± 47.63^{a}	6.24 ± 0.24^{b}	0.96 ± 0.02^{c}
GH	893.42 ± 42.11^{a}	882.68 ± 43.83^{a}	6.28 ± 0.20^{b}	0.96 ± 0.01^{bc}
I	884.42 ± 55.49^{a}	877.09 ± 51.13^{a}	6.51 ± 0.23^{ab}	0.97 ± 0.00^{abc}

Note: Different lowercase letters indicated significant differences (P < 0.05) among different groups.

The β-diversity was used to evaluate the differences in the composition of the flora between the sample groups with the principal coordinate analysis (PCoA). Compared with the C group, the intestinal flora structure of the M group was seriously deviated, which indicated that gut microbiota dysbiosis occurred. Under the administration of SBSG, the changes in flora can be mitigated to varying degrees. The flora structure of the GH group had almost recovered to similar to the C group (Fig. 1A).

Furthermore, the changes in the abundance of gut microbiota composition in each group were analyzed. At the phylum level, it can be seen from Fig. 1B that Firmicutes and Bacteroidetes account for the majority of the flora composition in each group. The changes in

the ratio of Firmicutes to Bacteroidetes (F/B) are often used to indicate the occurrence of gut microbiota dysbiosis[19-20]. It can be seen that compared with the C group (Fig. 1C), the F/B ratio of the M group decreased, and the F/B ratio was up-regulated to varying degrees after the SBSG intervention. The GM group in the highest degree of up-regulation, but there was no significant difference in statistics. It had been indicated that dysbiosis under this condition did not change the Firmicutes and Bacteroidetes which accounted for a large proportion. From the results of Fig. 1C, it can be seen that the proportion of Proteobacteria and Patescibacteria increased significantly after arsenic exposure ($P < 0.05$). SBSG could down-regulate their abundance. Verrucocrobiota, a small proportion in the phylum, was almost absent in arsenic exposure, only expressed in SBSG and I groups, and it showed a dose-dependent increase after SBSG intervention.

At the genus level, it can be seen that the overall composition of intestinal flora in each group of mice is similar at the genus level (Fig. 1D). There are differences in some specific bacteria as can be seen from Fig. 1E, arsenic exposure up-regulated *Alistipes*, *Bacteroides*, *Clostridia*_UCG-014, *Muribaculum*, *Anaeroplasma*, *Candidatus_Saccharimonas*, especially of *Alistipes* and *Candidatus_Saccharimonas* ($P < 0.05$). Furthermore, it down-regulated the abundance of *Ruminococcus* and *Akkermansia*. Under SBSG intervention, the genus changes caused by arsenic were reversed in different degrees. It can be seen that compared with the M group, the GL and GM groups were dose-dependently down-regulated by arsenic-induced bacterial changes. In contrast, the GH group's intervention was slightly lower than the other 2 doses of the SBSG group. In general, the intervention effect of the GM group on down-regulating harmful bacteria was the best. Compared with the M group, *Alistipes*, *Bacteroides*, *Muribaculum*, *Anaeroplasma*, and *Candidatus_Saccharimon* were significantly down-regulated by 31.06%, 48.75%, 46.07%, 72.01% and 47.93% respectively in GM group. *Ruminococcus* and *Akkermansia*, which were down-regulated by arsenic, improved after SBSG treatment, but only significantly up-regulated in GM and GH groups respectively ($P < 0.05$).

Linear discriminant analysis effect size (LEfSe) was used to reveal the most dominant species that led to the difference in the structure of the mice in each group. Combined with LEfSe evolutionary branch diagram and LDA value distribution histogram (Fig. 2A), it can be seen that the differential markers *Anaeroplasma*, *Alistipes, Bacteroides*, *Turicibacter* and *Candidatus_Saccharimonas* in the M group at the genus level were mostly potential pathogens[21-22]. The differential markers *Bifidobacterium*, *Ruminococcus*, *Dubosiella*, *Erysipelatoclostridium*, *Akkermansia* and Lachnospiraceae_UCG_006 in the SBSG groups were mostly common probiotics[23]. Therefore, this part can further explain that arsenic exposure can cause up-regulation of potential pathogens, and SBSG can interfere with gut microbiota dysbiosis by enriching specific probiotics.

The changes in gut microbiota are often accompanied by changes in various functional pathways. Thus, the PICRUSt2 functional prediction model was used to analyze and speculate the functional gene composition of each sample. It can be seen that arsenic exposure

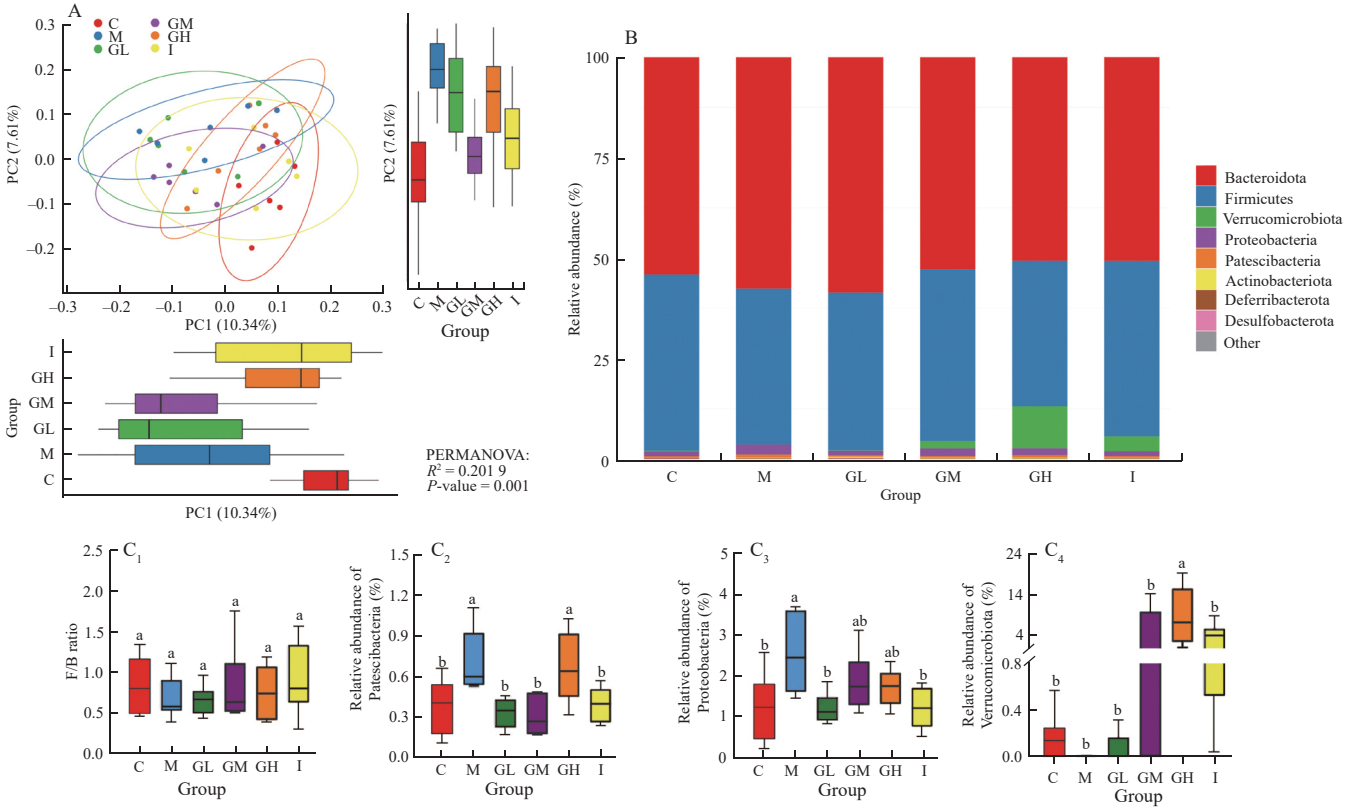

Fig. 1 Effects of SBSG on gut microbiota in arsenic-induced mice. (A) PcoA analysis of β-diversity. (B) Abundance map of gut microbiota composition (phylum level). (C) Community composition at the phylum level. (C_1) Relative abundance ratio of F/B; relative abundance of (C_2) Patescibacteria, (C_3) Proteobacteria and (C_4) Verrucomicrobiota, respectively. (D) Cluster heatmap of gut microbiota (genus level) between groups. (E) Community composition at the genus level. Relative abundance of (E_1) *Alistipes*, (E_2) *Bacteroides*, (E_3) *Clostridia*_UCG-014, (E_4) *Muribaculum*, (E_5) *Anaeroplasma*, (E_6) *Candidatus_Saccharimonas*, (E_7) *Ruminococcus*, and (E_8) *Akkermansia*, respectively. Different lowercase letters indicate significant differences ($P < 0.05$) among different groups.

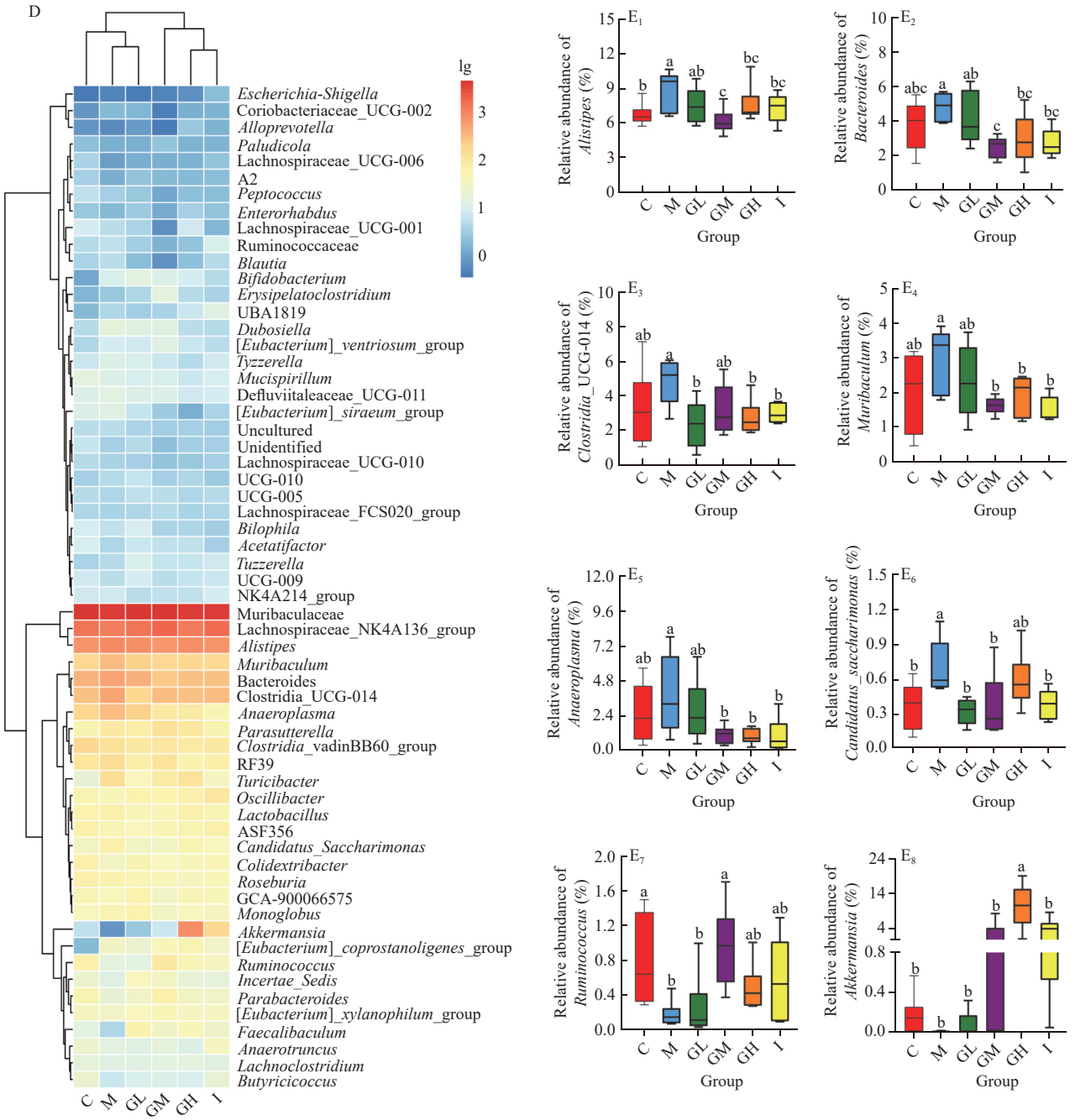

Fig. 1 (*Continued*)

would affect various metabolic genes (Fig. 2B), which significantly down-regulated steroid biosynthesis and carotenoid biosynthesis metabolism. After the regulatory of SBSG, these 2 metabolic pathway genes were up-regulated and gradually returned to the C group. Because SBSG is a kind of carbohydrate, its utilization by gut microbiota is closely related to the carbohydrate-active enzymes (CAZy)[24]. Therefore, based on the CAZy database, the prediction analysis was carried out. It can be seen that compared with the C group, the glycoside hydrolase GH84 and glycosyl transferase GT10 in the M group were significantly down-regulated, GT19 was slightly down-regulated, and GH16 was significantly up-regulated (Fig. 2C). This change was reversed under the regulatory of SBSG. Spearman correlation was used to analyze the correlation between bacteria and CAZy-related expression. The results showed that GT19 was positively correlated with *Akkermansia*, GH16 was positively correlated with [*Eubacterium*]_*coprostanoligenes*_ group and negatively correlated with *Clostridia*_vadinBB60_group (Fig. 2D). The prediction results of this part also clarify that SBSG not only improved the structure of the flora but also had a certain potential regulatory effect on the changes of related metabolic pathways, which needs further in-depth analysis.

3.2 *Effects of swim bladder sulfated glycosaminoglycan on SCFAs*

SCFAs are short-chain organic fatty acids produced by gut microbiota fermentation of carbohydrates, proteins, and some

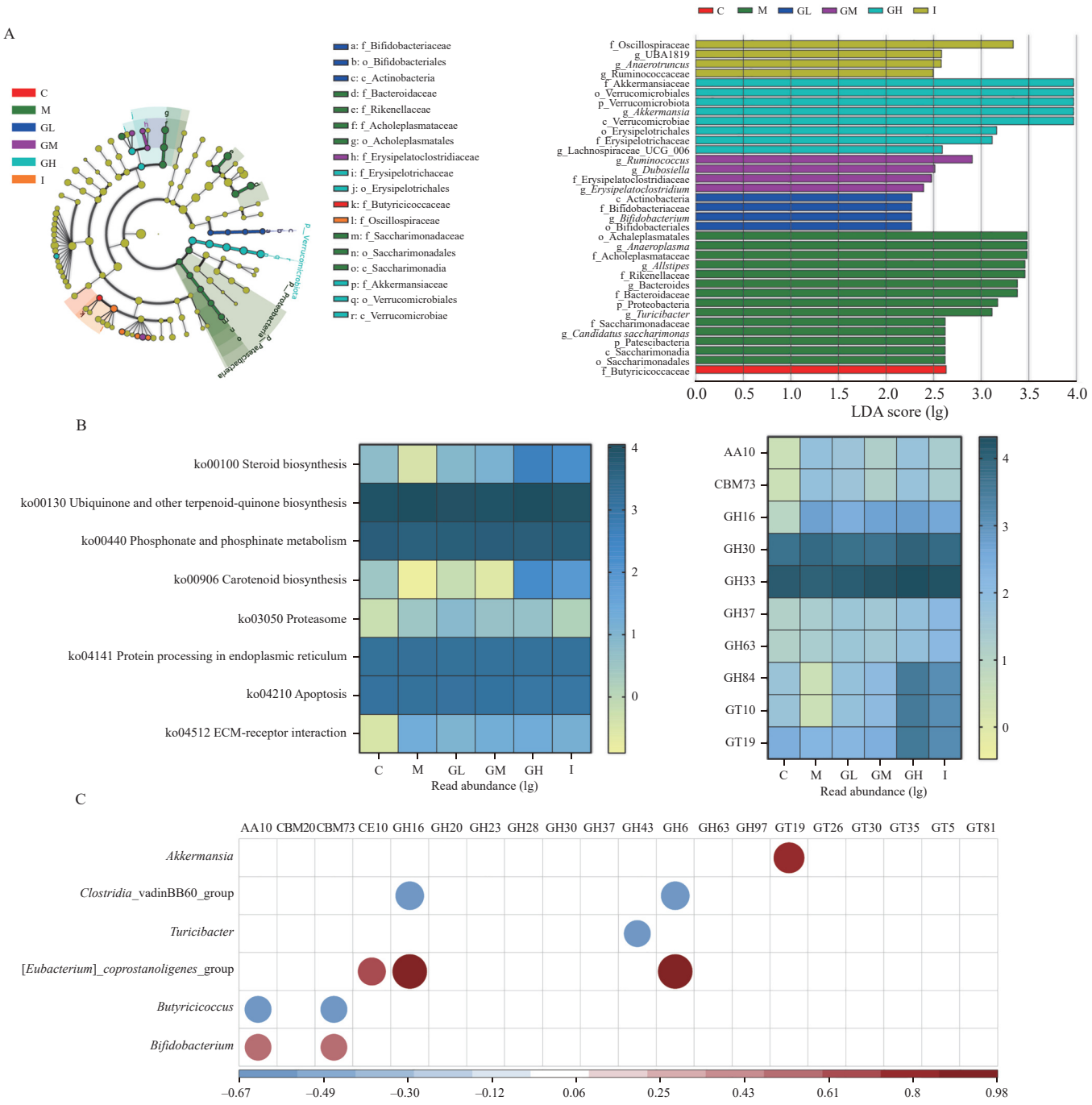

Fig. 2 Effects of SBSG on biomarker and potential functional changes in arsenic-induced mice. (A) Biomarkers of mice by LEfSe analysis (LEfSe evolutionary branch diagram and LDA value distribution histogram). (B) The heatmap of difference in KEGG metabolic-related genes and CAZy based on PICRUSt2 prediction. (C) The correlation between differential genus and CAZy using Spearman rank correlation. The heatmap showed that red was positively correlated and blue was negatively correlated. The size and color of the circle were related to the degree of correlation.

endogenous substrates that are not digested by the upper digestive tract. SCFA production plays an important role in regulating functional metabolism[25]. In this experiment, the content of SCFA in cecal and colonic feces was measured to evaluate the effects of SBSG on arsenic exposure by adjusting intestinal structure to produce beneficial metabolites.

From Figs. 3A and B, it can be seen that compared with the C group, the total SCFA in the cecal and colonic feces of the M group showed a downward trend, indicating that arsenic exposure led to gut microbiota dysbiosis and further affected the reduction of beneficial metabolites. However, the total SCFAs in the colonic feces of each SBSG dose group showed an upward trend, and the total SCFA content was the highest in the GH group. It can show the changes of a single SCFA in the cecal and colonic feces of each group of mice in Figs. 3C-I. Compared with the M group, the SCFA of the cecal feces of mice in each group of SBSG showed a downward trend, except for hexanoic acid. The I group showed the same trend, and there was a significant down-regulation of propionic acid and butyric acid. Propionic acid is mainly involved in gluconeogenesis in the liver, while butyric acid is the main energy source for colon cells[26]. This phenomenon indicated that SBSG

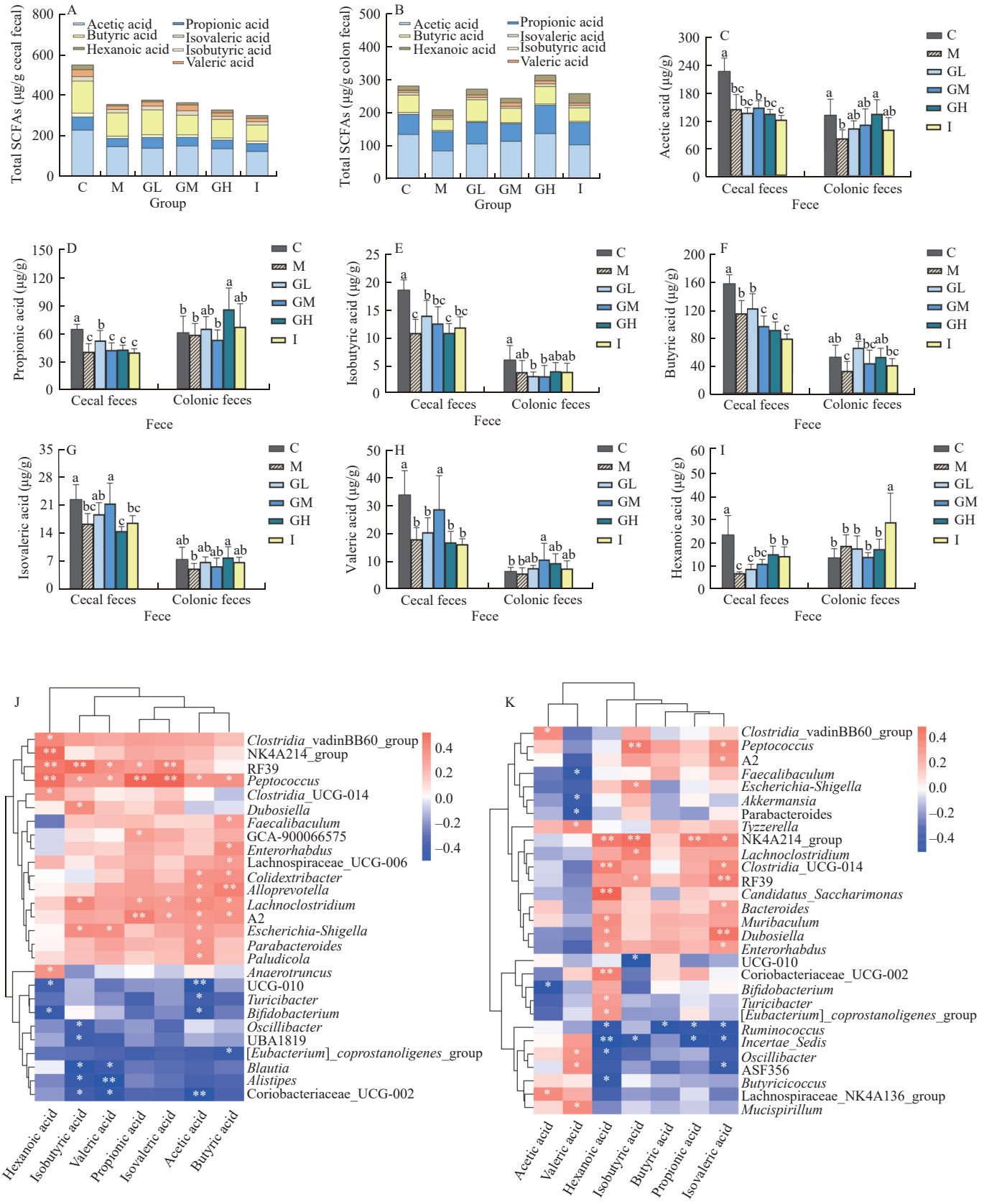

Fig. 3 Effects of SBSG on SCFA production in arsenic-induced mice. Total SCFA production in (A) cecum and (B) colon. (C) Acetic acid, (D) propionic acid, (E) isobutyric acid, (F) butyric acid, (G) isovaleric acid, (H) valeric acid, and (I) hexanoic acid contents in cecal feces and colonic feces. Different lowercase letters indicate significant differences ($P < 0.05$) among different groups. The heatmap representation of correlation for gut microbiota and SCFA in (J) cecal feces and (K) colonic feces. Red represented a positive correlation, blue represented a negative correlation. *$P < 0.05$, **$P < 0.01$.

may accelerate the utilization efficiency of SCFA at the front end of the lower digestive tract and promote its utilization by various organs and peripheral tissues.

In the SCFA analysis of colonic feces, it was concluded that SBSG was further decomposed and utilized by gut microbiota to produce more SCFA after reaching the colon. Compared with the M group, SBSG dose-dependently increased the acetic acid, and the GH group was significantly up-regulated by 64.28%. For other SCFA, it also showed different degrees of up-regulation trend, and the overall effect of the GH group was the best. The up-regulation level was close to or higher than that of the C group. These results further illustrated that SBSG can be used by the gut microbiota to ferment after reaching the colon and showed the same characteristics as inulin, which is a potential prebiotic to intervene in arsenic-induced gut microbiota dysbiosis in mice.

3.3 Correlation analysis between gut microbiota and SCFAs

The production of SCFA is closely related to the structure of gut microbiota. Therefore, the correlation between gut microbiota and SCFA production was further analyzed by Pearson correlation analysis[27]. It can be seen from the heatmap of Fig. 3J that most of the genera in the correlation analysis of cecal feces are not the bacteria that account for a large proportion or change significantly in the genus analysis. As shown in Figs. 2E, 3G and H, significantly changed genus *Alistipes* was negatively correlated with the production of valeric acid and isovaleric acid, which is consistent with the previous determination of SCFA content in cecal contents. In the analysis of colonic feces (Fig. 3K), all the significantly changed genera showed a correlation with SCFA production in the colon except for *Anaeroplasma*. In particular, it can be noted that *Candidatus_Saccharimonas* showed a significant positive correlation with the production of hexanoic acid and a negative correlation with acetic acid, valeric acid, and butyric acid, which was consistent with the results of SCFA determination in colonic feces. From these results, it can be seen that the production of SCFA is not only related to the enrichment of individual dominant bacteria but also to the overall flora structure.

3.4 Effect of swim bladder sulfated glycosaminoglycan on short-chain fatty acid-related receptors

SCFA can activate some specific receptors to regulate body function, such as G protein-coupled receptor 43 (*GPR43*), *GPR109A*, and olfactory receptor 78 (*Olfr78*), which have been reported to be associated with colitis, arthritis, and metabolic function regulation[28]. Arsenic exposure significantly down-regulated the gene expression of these receptors (Figs. 4A-C). Compared with the C group, it down-regulated *GPR43*, *GPR109A*, and *Olfr78* gene expression by 35.18%, 40.93%, and 49.74%, respectively. After the intervention of SBSG, the expression of SCFA receptors was up-regulated to varying degrees. It can be shown that compared with the M group, *GPR43* was temporarily down-regulated in the GL group, and its expression was gradually significantly up-regulated with the increase of dose, and in the GH group was nearly 1 times higher than that of the M group (Fig. 4A). It can be seen that compared with the M group, the GH group showed a significant increase in the expression of *GPR109A* ($P < 0.05$) and up-regulated *Olfr78* ($P > 0.05$). From this part, it can be known that SBSG mediated SCFA to up-regulate the expression of SCFA-related receptors *GPR43* and *GPR109A* to interfere with the damage caused by arsenic exposure.

3.5 Effects of swim bladder sulfated glycosaminoglycan on genes related to physical, chemical, and immune barrier

The intestinal barrier can be divided into biological, physical, chemical, and immune barriers[6]. From the data mentioned above, it can be seen that arsenic exposure leads to the destruction of the biological barrier. Therefore, RT-qPCR was used to further analyze the changes in physical, chemical, and immune barrier-related genes. The intestinal physical barrier is mainly composed of intestinal epithelial cells (IECs) and tight junctions (TJs)[29]. In this experiment, *claudin-1*, *occludin*, and *ZO-1*, which are important parts of TJs, were determined. It can be seen from Figs. 5A-C that compared with the M group, the GL and GM groups significantly up-regulated the *occludin* and *ZO-1*, but there was no significant difference in the GH group. SBSG also up-regulated the gene expression of *claudin-1* but did not exist significant difference compared with the M group ($P > 0.05$).

The intestinal chemical barrier is mainly composed of antimicrobial substances, digestive enzymes, and mucins. As the skeleton of the mucus layer, mucin is not only a part of the chemical barrier but also an important component of the physical barrier[30]. Mucin 1 (*MUC-1*) and *MUC-2* play an important role in the chemical barrier. Compared with the M group, the GL group significantly up-regulated *MUC-1*, while the opposite trend was observed for *MUC-2* (Figs. 5D and E). This offset effect may also lead to a smaller intervention effect of the GL group on arsenic-exposed mice. The

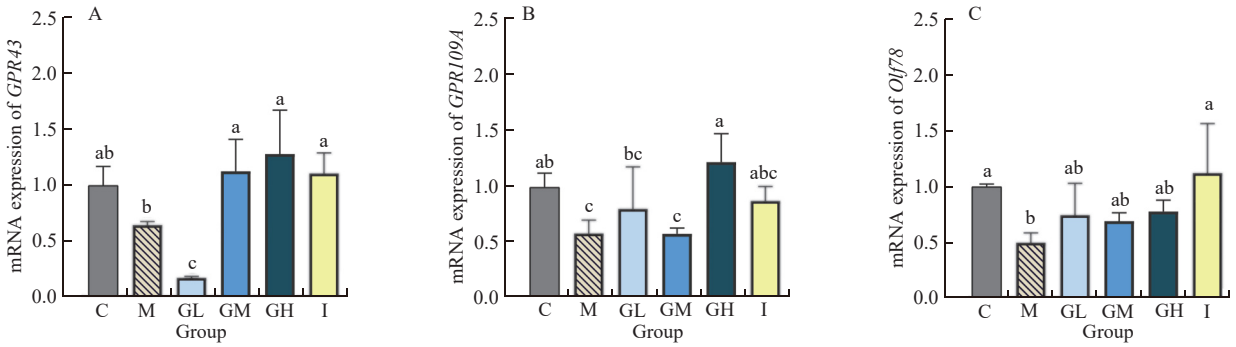

Fig. 4 Effects of SBSG on SCFA-related receptors in arsenic-induced mice. The relative gene expression of SCFA-related receptors in the colon including (A) *GPR43*, (B) *GPR109A*, and (C) *Olf78*. Different lowercase letters indicate significant differences ($P < 0.05$) among different groups.

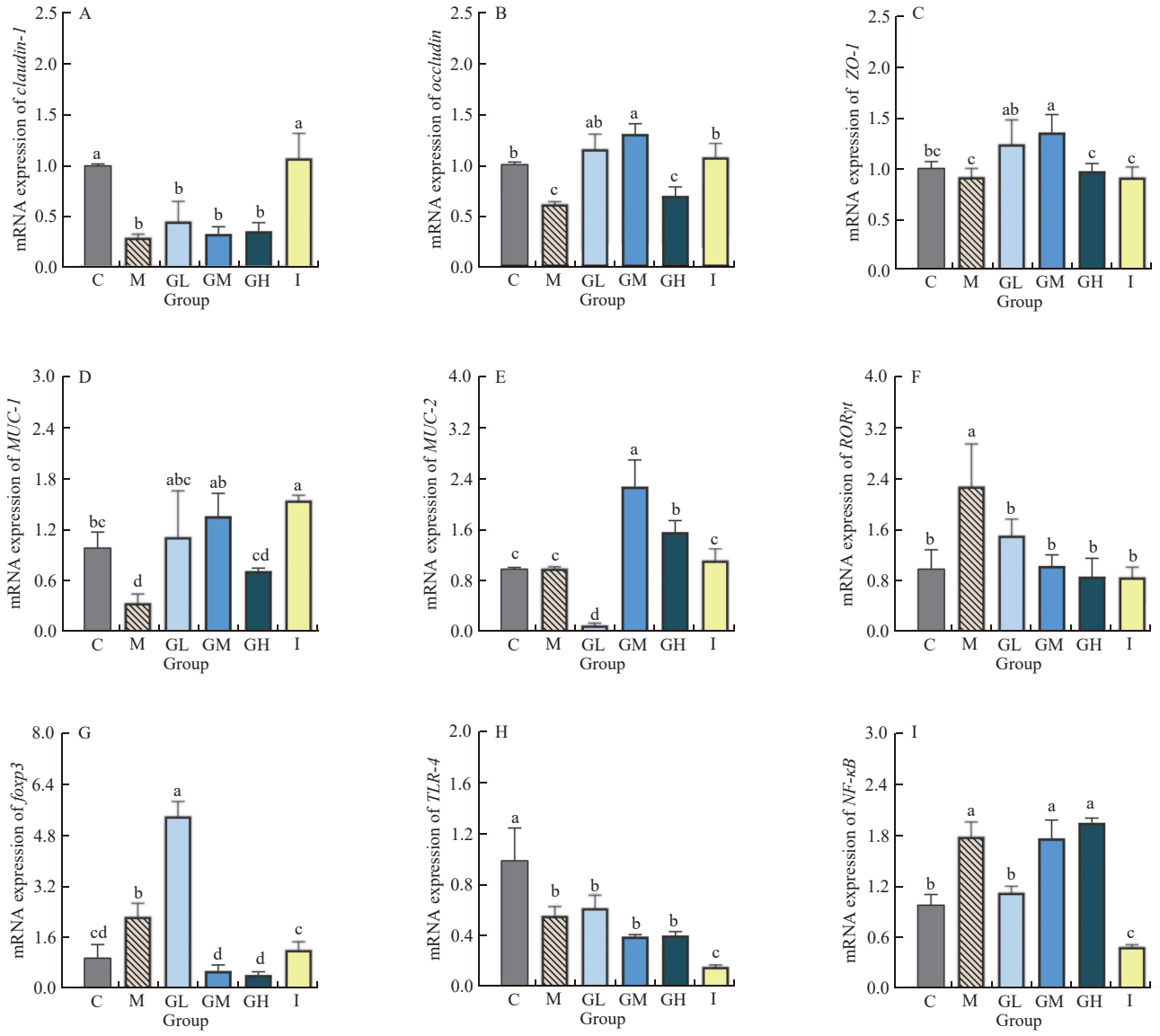

Fig. 5 Effects of SBSG on intestinal barrier in arsenic-induced mice. The relative gene expression of the physical barrier in the colon includes (A) *claudin-1*, (B) *occludin*, and (C) *ZO-1*. The relative gene expression of chemical barriers in the colon includes (D) *MUC-1* and (E) *MUC-2*. The relative gene expression of the immune barrier in the colon includes (F) *RORγt*, (G) *Foxp3*, (H) *TLR4*, and (I) *NF-κB*. Different lowercase letters indicated significant differences ($P < 0.05$) among different groups.

significant up-regulation of *MUC-1* and *MUC-2* in the GM group was 2.92 and 1.27 times, respectively. However, the GH group showed a downward trend compared with the GM group.

The intestinal immune barrier is mainly composed of immune cells and immune factors. $CD4^+$ T cells, which mainly include Th1, Th2, Th17, and Treg, are a subset of cells that play an important role in immunity. The balance of Th17/Treg cells is often used to evaluate the stability of intestinal immunity in many studies. Therefore, we evaluated the intestinal immune balance by measuring the main transcription factors *RORγt* and *Foxp3* of Th17 and Treg cell differentiation[31]. The expression of *RORγt* and *Foxp3* was significantly up-regulated after arsenic exposure compared with the C group (Figs. 5F and G). Compared with the M group, the expression of *RORγt* was significantly down-regulated in GL, GM, and GH groups by 33.60%, 54.7%, and 61.52%, respectively. There was no significant difference between each group and the I group. For the expression of the *Foxp3*, there was a brief significant up-regulation at the GL group, and it was down-regulated with the increase in dose. The effect of GM and GH groups was better than that of the inulin. It can be concluded that SBSG can alleviate arsenic-induced intestinal immune imbalance by regulating Th17/Treg cell balance. As a polysaccharide, SBSG has direct and indirect regulatory forms for intestinal immunity. In the direct regulatory form, it mainly depends on the mutual recognition and interaction with immune cell receptors. Among them, toll-like receptor 4 (*TLR4*) is a key pattern recognition receptor, and its activation will initiate the NF-κB signaling pathway[32]. Therefore, this experiment also explored the direct immunomodulatory effect of SBSG by measuring the expression of *TLR4* and *NF-κB*. It can be seen from Fig. 5H that compared with the M group, there was no significant difference in the expression of *TLR4* in each SBSG group, but it showed a downward trend in the I group. Arsenic exposure significantly up-regulated the expression of the *NF-κB*, and

the GL group was significantly down-regulated to the level of the C group, while the GM and GH groups showed an upward trend, and the I group was more down-regulated than the GL group (Fig. 6I). These results indicated that *TLR4* and *NF-κB* gene expression did not show a correlation, and it needed to be further verified whether TLR4/NF-κB signaling pathways were affected by the SBSG direct regulatory role.

3.6 Effect of swim bladder sulfated glycosaminoglycan on the expression of TJ protein

To further explore the effect of SBSG on the intestinal physical barrier of arsenic-induced gut microbiota dysbiosis mice, the protein expression was further determined based on gene analysis. Arsenic exposure significantly down-regulated the protein expression of claudin-1 and ZO-1, but there was no significance of occludin (Fig. 6A). After SBSG intervention, the expression of TJs was up-regulated to varying degrees. In the regulation for claudin-1, the GL, GM, GH and I groups were up-regulated by 0.84, 1.33, 0.75, and 0.47 times compared with the M group, respectively. In the regulation for ZO-1, the GL, GM, GH and I groups were up-regulated by 0.52, 0.79, 0.42 and 0.94 times, respectively. In the regulation for *occludin*, only the GL group showed significant differences. These results indicated that SBSG interfered with arsenic-induced physical barrier damage by up-regulating the expression of TJs, and the intervention effect was best in the GM group.

3.7 Effects of swim bladder sulfated glycosaminoglycans on the expression of immune barrier-related proteins

In the analysis of the gut microbiota, it was found that arsenic exposure induced an up-regulation of the abundance of inflammation-related bacteria. In the gene determination, it was found that SBSG could interfere with the genetic changes of TLR4 and NF-κB caused by arsenic, but the correlation was not explored. Therefore, in this part, protein expression was used to verify the intervention effect of SBSG on the TLR4/NF-κB signaling pathway which connected to inflammation and immunity. It can be seen that arsenic exposure can significantly promote the up-regulation of the TLR4 and its adaptor receptor MyD88, while the expression of these 2 receptor proteins was significantly reduced after SBSG intervention (Fig. 6B). Furthermore, it can be seen that SBSG also significantly inhibited the phosphorylation of IKβα and p65 which were downstream proteins of the NF-κB signaling pathway. SBSG regulation of IKβα was similar to that of TLR4, and the effect of the GM group was the best. On the protein expression of p65, with the increase of SBSG dose, it showed a certain upward trend but still showed an inhibitory effect compared with the M group. It can be concluded that at the protein level, SBSG interfered with the immune barrier damage by inhibiting the activation of the TLR4/MyD88/NF-κB signaling pathway.

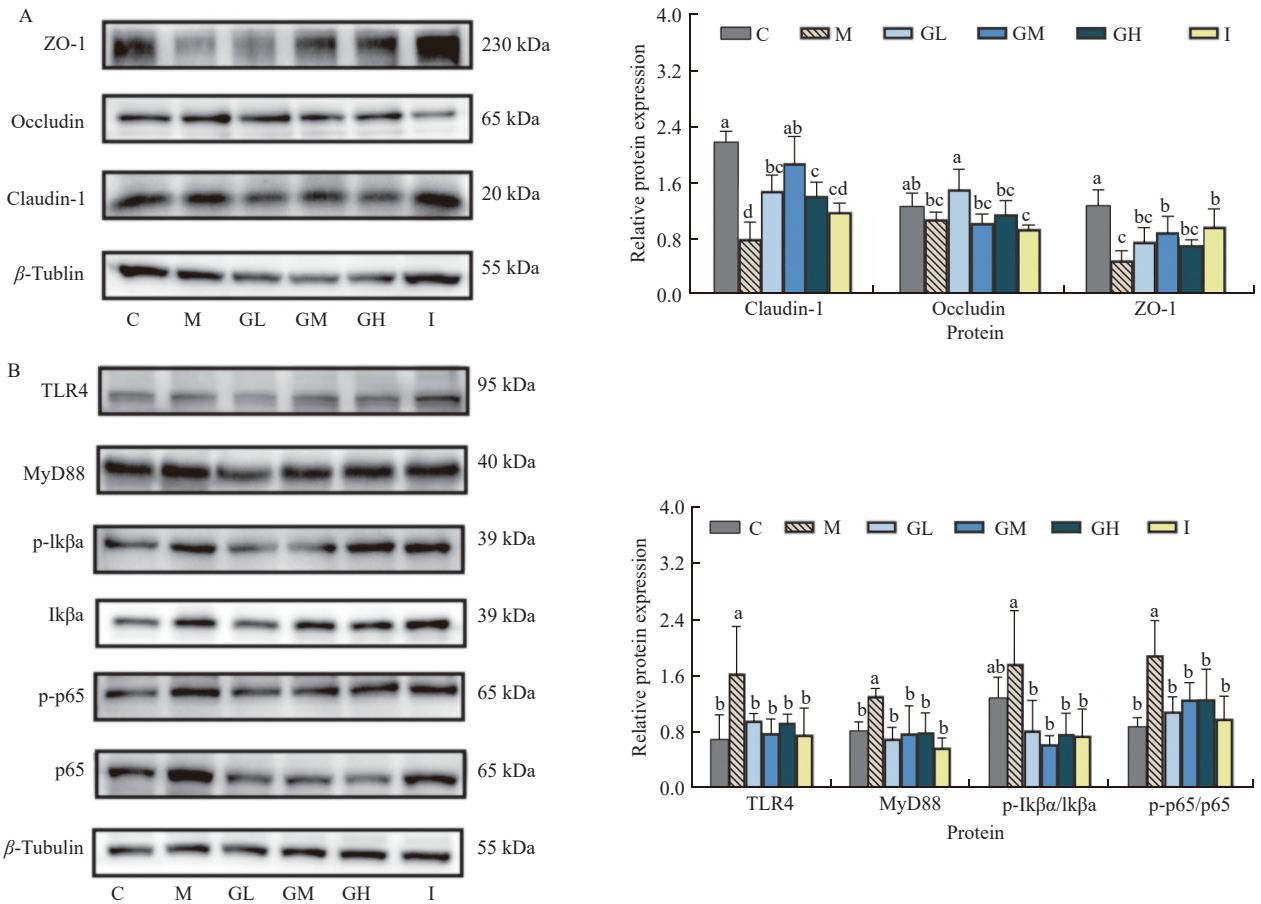

Fig. 6 Effects of SBSG on intestinal barrier-related protein in arsenic-induced mice. (A) Image of Western blot and its relative protein expression of TJ protein. (B) Image of Western blot and its relative protein expression of TLR4/MyD88/NF-κB signaling pathways. Different lowercase letters indicated significant differences ($P < 0.05$) among different groups.

4. Discussion

Arsenic can induce intestinal epithelial cell damage which will break the integrity of the intestinal barrier. Thus gut microbiota is more likely to transfer from the original position to other positions, resulting in gut microbiota dysbiosis. Based on the previous research[16], SBSG was used to explore its regulatory effect on arsenic-induced gut injury *in vivo*.

In the analysis of gut microbiota in this experiment, through *α*-diversity, *β*-diversity, flora abundance analysis, key biomarker analysis, and functional prediction, it can be indicated that short-term exposure to 5 mg/kg sodium arsenite for 38 days had little effect on *α*-diversity, but significantly affected *β*-diversity. Similar results were also presented after 6 months of exposure to 50 mg/kg arsenic[33]. At the phylum level, arsenic will lead to an increase in the abundance of Bacteroidetes and a decrease in the abundance of Firmicutes. This is due to the presence of arsenic tolerance genes in the Bacteroidetes and the lack of them in the Firmicutes[34]. After the intervention of SBSG, the decrease in the F/B ratio induced by arsenic was reversed. At the phylum level, Proteobacteria, Patescibacteria, and Verrucocrobiota had significant changes. Among them, Proteobacteria contains more conditional pathogenic bacteria[34]. The increase in its abundance is often an important indicator of dysbiosis, and it also has a certain positive correlation with intestinal permeability and is considered to be the iconic phylum of colitis[20]. The increase of Patescibacteria has been reported to be closely related to oxidative stress and inflammatory response caused by liver injury[35]. The above 2 potentially harmful bacteria with a significant down-regulation in the SBSG group. Verrucocrobiota, as a relatively small proportion of flora, the significant change in this experiment was caused by the change of *Akkermansia*. In recent years, *Akkermansia* is a new type of probiotic, which has shown beneficial effects on enhancing the mucosal barrier, promoting SCFA production, alleviating heavy metal toxicity, and regulating colitis[10,36]. Arsenic exposure reduced its abundance and was close to non-expression. While it increased in a dose-dependent manner in the SBSG group, it can be shown that SBSG had the effect of promoting the growth of probiotics. Further analysis of the genus showed that *Alistipes*, *Bacteroides*, *Clostridia*_UCG-014, *Muribaculum*, *Anaeroplasma*, and *Candidatus_Saccharimonas* that were upregulated after arsenic exposure were mostly potential pathogens. Among them, *Alistipes* is associated with cancer, cardiovascular disease, and colitis[37]. *Candidatus_Saccharimonas* has been reported to be positively correlated with the incidence of colon cancer and was a pro-inflammatory bacteria, which had a certain potential immunosuppressive effect[22,38]. After the intervention of SBSG, the above potential pathogenic bacteria showed different degrees of down-regulation. For the arsenic-induced down-regulated genus *Akkermansia* and *Ruminococcus*, the former is known as a well-known probiotic substance from the previous article, while *Ruminococcus* showed adverse effects in diarrhea, intestinal diseases, and liver damage[39-40]. However, it has also been shown that it would show a downward trend in heavy metal exposure and had the effect of enhancing the mucosal barrier and producing the beneficial substance SCFA, so *Ruminococcus* may be a genus with both advantages and disadvantages[41-42]. In the analysis of biomarkers, it can also be shown that the biomarkers in the M group were mostly harmful bacteria, while the biomarkers of the SBSG groups, *Bifidobacterium*, Lachnospirceae_UCG_006, and *Akkermansia*, were common probiotics. Flora analysis showed that SBSG almost reversed the arsenic-induced changes in the flora structure and gradually tended to the C group, and it has a similarity with the prebiotic inulin. Based on the changes in flora, its function was predicted. It was found that arsenic exposure significantly down-regulated steroid biosynthesis and carotenoid biosynthesis metabolic genes. The former was mostly related to growth and development and lipid metabolism, while the latter was related to oxidation and the immune system[43-44]. From the prediction results, it can be inferred that arsenic exposure leads to dysbiosis accompanied by metabolic disorders. After SBSG intervention, it can effectively regulate the flora and restore the metabolic balance. The various metabolites produced by SBSG under the action of gut microbiota may have some substances that can participate in promoting the transformation of arsenic to exert a direct role in detoxification. In subsequent studies, non-targeted metabolomics can be used to further explore the metabolic that are significantly changed in SBSG intervention to explore its direct detoxification and establish the interaction network between the intestine and various organs. In functional prediction, CAZy was produced by specific gut microbiota. Arsenic exposure can cause CAZy disorder, and the main reason for the change is related to the abundance of gut microbiota. Under the action of CAZy, polysaccharides were metabolized to produce SCFA.

In this experiment, the production of SCFA in cecal feces and colonic feces was measured respectively. It was found that gut microbiota dysbiosis caused by arsenic exposure, which made the SCFA produced by the fermentation of SBSG in the front of the large intestine to a greater extent metabolized to the liver, blood, or intestinal epithelial cells to resist the adverse effects, so it can be measured that the unused SCFA were less. After reaching the colon, it was fully fermented by the gut microbiota to produce more SCFA, so the content of each SBSG group was significantly increased, and the role of this part of SCFA in the intestine was more to maintain the integrity of the intestinal barrier. These results further verified that SBSG has potential prebiotic characteristics. Through the analysis of gut microbiota and SCFA content, it was also found that there was a certain correlation between the significantly changed gut microbiota and the production of SCFA in the colon, especially *Candidatus_Saccharimonas*. The change in it and the production of SCFA showed a clear consistency. However, it can also be seen that some harmful bacteria caused by arsenic exposure were positively correlated with the production of some SCFAs, which may be one of the reasons why SBSG has no significant regulatory effect on some SCFAs. This part also showed that there is no obvious boundary between beneficial bacteria and harmful bacteria, while the structure of gut microbiota plays an overall role. There are some floras with arsenic resistance genes and detoxification in organisms, and studies have shown that the cell wall polysaccharides and transporters of probiotics can complex heavy metals. In addition, some flora also play a role in promoting enzyme and defense responses[45]. From our results, we can speculate that SCFA exerts an indirect detoxification effect by regulating the structure of the flora.

The production of SCFA will activate some specific receptors, such as *GPR43*, *GPR109A*, and *Olf78*. The activation of these receptors has a certain impact on maintaining the intestinal barrier, body metabolism, and immune health[26]. In general, each group of SBSG significantly promoted the expression of SCFA receptors. Upon determination of the SCFA content in the colon, it was

demonstrated that, although SBSG did not exhibit a significant impact on certain SCFAs, it had a notable effect on the upregulation of the genes encoding the relevant receptors. This may be due to the binding of SBSG to the receptor, which in turn promotes the expression of SCFA-related receptors.

Intestinal barrier integrity is a key factor in determining the stability of microecology[6]. SBSG showed a certain up-regulation effect on the gene and protein expression of claudin-1, occludin, and ZO-1, which are important substances that constitute the physical barrier. In our previous study, we found that SBSG can mediate the JNK signaling pathway to up-regulate the expression of ZO-1 *in vitro*, and in this part of the *in vivo* experiment, we can also draw the regulation of SBSG on TJ protein in dysbiosis mice. The SBSG role *in vivo* may be related to its characteristics, the enriched gut microbiota, the produced metabolites, or the activated SCFA receptors. In the composition of the physical barrier, the mucus layer and mucin are sometimes included. MUC-1 is a transmembrane mucin present in the intestinal epithelium, while MUC-2 is a secreted mucin secreted by goblet cells. Both play an important role in maintaining the stability of the mucosal structure and are also part of the chemical barrier. After the determination, it was found that arsenic exposure had a significant effect on MUC-1 but a small effect on MUC-2, which reflected that arsenic exposure had a significant effect on intestinal epithelial damage. Under the intervention of SBSG, both MUC-1 and MUC-2 showed an up-regulation effect at a specific dose. On the one hand, the up-regulation of mucin may be due to the enrichment of some intestinal flora, such as *Akkermansia*, which is famous for promoting mucin production. On the other hand, it may also be attributable to SBSG itself, acting as a glycosaminoglycan, binding to proteins to form glycoproteins and interacting with mucins. In terms of immune barrier integrity, this study measured the intestinal immune balance by the gene expression of Th17/Treg transcription factor RORγt/Foxp3. The differentiation of Th17 will induce a pro-inflammatory response, while Treg cells play an opposite role. In general, the differentiation of Th17 will be inhibited while the differentiation of Treg will be promoted[46]. However, in this experiment, it can be seen that the RORγt and the Foxp3 were significantly up-regulated after arsenic exposure. This result also appeared in the study of Chen et al.[31], and there may be some factors that affected the immune balance and promoted the differentiation of Treg at the same time. From the analysis of RORγt/Foxp3, it can be shown that arsenic would eventually break the dynamic balance of Th17/Treg cells, which is more biased towards the differentiation of Th17 to promote immune inflammatory response. In the previous flora analysis, most of the harmful bacteria up-regulated by arsenic exposure were some pro-inflammatory bacteria, which were related to the results of this part. SBSG could reduce the expression of RORγt and Foxp3 in different degrees and alleviate the imbalance of Th17/Treg. From the perspective of direct immune receptor regulation, SBSG had a certain effect on the immune barrier induced by arsenic at the genetic level, but the overall trend did not show obvious regularity. However, in terms of protein expression, it can be seen that arsenic exposure significantly activated the expression of TLR4 and its adaptor protein MyD88 and the upstream and downstream key proteins of the NF-κB signaling pathway. SBSG significantly inhibited these changes and exerted good anti-inflammatory properties.

Based on the above differences in the results of gene and protein expression, the reason could be illustrated from the perspective of epigenetics, which explains that chromosomes, DNA or RNA may be modified during gene transcription so that RNA can be transcribed smoothly but not translated to proteins[47]. In recent years, it has also been found that RNA can be modified by glycosylation[48]. The presence of SBSG will have a certain effect on the transcription process of RNA. Besides, the reason could also be that SBSG is caused by its characteristics. Glycosaminoglycans are substances that could bind to core proteins to form glycoproteins under the action of various enzymes to regulate body function. It is well known that substances such as the SCFA-related receptors, TJs, mucins, TLR4, and MyD88 are proteins. Therefore, it can be reasonably speculated that SBSG can bind to related receptors to promote the expression of related proteins, leading to differences in gene expression. However, the specific role still needs to be proved through molecular docking or other means. Based on the above results of each part of the intestinal barrier, it can be concluded that SBSG could effectively enhance the intestinal barrier to alleviate arsenic-induced gut injury. Black raspberries[49], *Flammulina velutipes* polysaccharides[50], galactooligosaccharide[12], and other substances with antioxidant or prebiotic properties[51-52] have been shown to have better heavy metal detoxification effects. In this experiment, SBSG can play a similar role as the above substances but also exert its novelty and innovative potential. Firstly, SBSG is derived from swim bladder, which is an active substance prepared in the laboratory. As a processing by-product, the high-value utilization of swim bladder has been low. Our research group obtained high-purity glycosaminoglycans by extraction, separation, and purification. In addition, some of its *in vitro* properties have been explored, but the *in vivo* properties have not yet been included. Therefore, this manuscript plays an important role in promoting the high value of swim bladder resources. Moreover, SBSG exhibited different regulatory properties due to its nature as a macromolecular GAG. It can be shown that this regulatory process mediated an indirect effect, which is produced by SBSG regulating the gut microbiota. It up-regulated beneficial bacteria, down-regulated harmful bacteria, restored the structure of gut microbiota, and produced favorable metabolites in the colon to further restore the intestinal barrier. Additionally, SBSG may also mediate a direct effect, by activating the relevant receptors and exerting its antioxidant and anti-inflammatory properties to enhance the intestinal barrier to relieve injury. Compared with the I group, SBSG had similar or better regulatory effects. Among the indicators, it was indicated that the dose of 100 mg/kg SBSG (GM group) had the best effect on enhancing the intestinal barrier, while the dose of 200 mg/kg SBSG (GH group) had more obvious enrichment of probiotics. SBSG not only overcame the limitation of glycosaminoglycan's macromolecules but also supplemented the heavy metal detoxification pathway. In future studies, the interaction between SBSG and specific probiotics can be explored and focus on the binding relationship between its structure and related receptor proteins, to further elucidate the target of SBSG in improving intestinal health. Moreover, whether SBSG also regulates other organs through the intestinal tract for heavy metal detoxification is also worth exploring.

Based on the above results, it can be concluded that SBSG played a potential prebiotic mechanism to interfere with arsenic-induced gut injury by regulating gut microbiota, metabolites, and intestinal barrier integrity, showing its great potential to become a new generation of prebiotics. These findings suggest that SBSG can be used to regulate related diseases caused by gut microbiota dysbiosis and a dietary supplement or drug additive for heavy metal detoxification.

Conflict of interest

The authors have declared no conflicts of interest.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250248.

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