Glucose oxidase exerts protective effects against Salmonella typhimurium infection by regulating the intestinal structure, immune response and cecal microbiota in murine

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Abstract

Glucose oxidase (GOD), an aerobic dehydrogenase, specifically catalyzes the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide, which are harmful to pathogenic bacteria but promoting the survival of beneficial bacteria in the intestine. Meanwhile, the increased antibiotic resistance induced by *Salmonella typhimurium* (ST) infections has amplified the need for development of novel therapeutic agents. As such, GOD may play an important role in controlling ST infections. To investigate the effects of GOD against ST infection, mice were pretreated with gentamicin (GM; positive control), GOD, or sterile water (negative control) and, four weeks later, challenged with ST or phosphate-buffered saline. Three days after infection, mice were sacrificed and samples were collected. In the present study, pretreatment with GOD or GM attenuated ST-induced body weight loss and mortality. Moreover, ST infection increased the intestinal damage, apoptosis in liver, inflammatory response in the colon and serum, which were reversed by GOD pretreatment. Sequence analysis further revealed that the gut bacterial community composition in mice pretreated with GOD was similar to that of the control group. Collectively, these findings indicate that GOD attenuates ST-induced negative effects by modulating intestinal barrier function, cytokine secretion, and intestinal microbiota composition in mice.

1 Introduction

*Salmonella typhimurium* (ST), a common gram-negative food-borne pathogen, has a wide host range, including humans and animals (Abatemarco et al., 2018). ST can cause a broad range of diseases from gastroenteritis and diarrhea to life-threatening systemic infections (Herrero-Fresno and Olsen, 2018), responsible for one million deaths per year (Balasundaram et al., 2017). Although numerous routes of infection have been described, oral transmission through contaminated water and food is the most common (Luvsansharav et al., 2020). ST proliferates in the intestine, adheres to the epithelial cells of the intestinal mucosa, invades the lamina propria, and releases toxins (Matamouros and Miller, 2015). Due to its metabolic diversity, *Salmonella* can colonize various surfaces as multicellular aggregates and increase the level of virulence through the formation of biofilms, which are often resistant to antibiotic treatment (Faccone et al., 2018; Ridge et al., 2018).

Currently, antibiotics continue to serve as an important means to treat *Salmonella* invasion, however, long-term, high-dose antibiotic regimens can alter the composition of the intestinal microbiota and further reduce human immunity (Gut et al., 2018). Hence, enhancing mucosal immune function and improving the stability of the intestinal microbiota are necessary to effectively combat ST infection. Moreover, the recent abuse of antibiotics has led to a significant increase in drug-resistant ST strains resulting in less effective preventative and therapeutic options (Siriken et al., 2020).

Glucose oxidase (GOD), an aerobic dehydrogenase fermented by *Aspergillus niger*, *Penicillium* and other fungi (Hatzinikolaou et al., 1996), specifically oxidizes β-D-glucose to gluconic acid and produces hydrogen peroxide (H₂O₂) while consuming a large amount of oxygen (Bankar et al., 2009). Owing to its broad functions, GOD is widely used in food and beverage processing, wound dressing, as well as other
biomedical applications, including the preparation of natural H$_2$O$_2$ antibacterial agents via reduction of nutrients (oxygen and glucose) required for bacterial growth (Bosiger et al., 2018; Tiina and Sandholm, 1989; Yeon et al., 2019). H$_2$O$_2$ is a commonly used fungicide with no obvious toxic effects, as it is spontaneously degraded into water and oxygen (Dubey et al., 2017; Finnegan et al., 2010; Linley et al., 2012; Maillard, 2002). Previous studies have indicated that GOD influences the intestinal environment by utilizing O$_2$ to produce gluconic acid and H$_2$O$_2$, which are harmful to pathogenic bacteria, however, promote the survival of beneficial bacteria (Kapat et al., 1998). Furthermore, GOD serves to maintain the ecological balance in the intestinal microcosm, improve the digestive environment and function of the gut, increase the abundance of beneficial bacteria, provide protection against oxidative stress, and improve growth performance and gut health (Asano et al., 1994; Cruz et al., 2012; Wu et al., 2019a).

The present study sought to investigate the protective effects of GOD pretreatment in mice in an effort to identify new preventative measures for ST infection, and provide a theoretical basis for the application of GOD in animal husbandry.

2 Materials And Methods

2.1 Experimental mice and reagents

C57BL/6 male mice (6-weeks-old) were obtained from Slac Animal Inc. (Shanghai, China) and housed at the Experimental Animal Center of Zhejiang University, China. Animal experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of Zhejiang University (ZJU2018-480-12) and were performed in accordance with relevant guidelines and recommendations, including the guide for the care and use of laboratory animal. The reporting follows the recommendations in the ARRIVE 2.0 guidelines. GOD powder (enzymatic activity is about 5,000 U/g) was prepared by the Feed Research Institute at the Chinese Academy of Agricultural Sciences (Beijing, China).

2.2 Experimental procedures

After two weeks of acclimation, forty-eight six- to eight-week-old male C57BL/6 mice (weighing approximately 20 g) were randomly allocated to six groups (n = 8/group) including control (CK), GOD, gentamicin pretreatment (GM), Salmonella typhimurium (ST), GOD + ST, and GM + ST groups. All mice were fed a basal diet (Slac Animal Inc., Shanghai, China) and their body weight was measured every 3 days. The mice in the CK and ST groups were fed sterile water, those in the GOD and GOD + ST groups were fed sterile water containing 0.1 U/mL GOD (20 U/kg body weight), and those in the GM and GM + ST groups were fed sterile water containing 0.4 mg/mL GM (80 mg/kg body weight) daily. On 28th day, the mice in the ST, GOD + ST, and GM + ST groups were administered 200 µL of S. typhimurium suspension (2 × 10$^9$ colony-forming unit (CFU)/mL) by oral gavage, while the mice in the CK, GM, and GM groups were administered the same volume of sterile phosphate-buffered saline (PBS). Body weight changes were measured on day 3 post challenge, and the mice were euthanized by cervical dislocation under anesthesia with diethyl ether. Samples were collected from the spleen, liver, blood, colon, cecum, and
ileum for analyzing histopathological features, bacterial translocation, gene expression, and inflammatory response.

Mucosa and tissue samples (1 g) were homogenized in 9 ml of 0.9% sterile saline (Aladdin, Shanghai, China) on ice and centrifuged at 3,000 × g for 15 min at 4°C. The total protein concentration of the supernatant was measured using a PierceTM bicinchoninic acid (BCA, No. A045-4-2) Protein Assay Kit, according to the manufacturer’s protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The prepared supernatant was stored at -80°C and used for testing.

2.3 Preparation of S. typhimurium culture

As described in a previous study, S. typhimurium CMCC 50115 (ST) were cultured and collected using standard batch culture procedures (Xu et al., 2018). A single ST colony was isolated from a streaked lysogeny broth (LB) agar plate and cultured in fresh LB medium in a shaking incubator at 37°C for 12–16 h. The overnight cultures were diluted at 1:5000 into 100 mL of fresh sterile LB medium. The cells were harvested in the logarithmic growth phase by centrifugation at 5000 rpm for 10 min, and subsequently washed three times with sterile PBS. The prepared ST bacteria were diluted with PBS to a concentration of 2 × 10^9 CFU/mL.

2.4 Hematoxylin and eosin (H&E) staining

During necropsy, ileal tissue samples were collected, fixed in 4% paraformaldehyde, dehydrated, and processed into paraffin sections according to the standard procedure. The paraffin sections were subjected to H&E staining for histopathological analysis (Xu et al., 2018).

2.5 Transmission electron microscopy

Ileal tissue samples were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) at 4°C for 4 h and then post-fixed in a mixture of 1% cold osmium tetroxide and 3% potassium ferrocyanide (1:1 v/v) for 2 h at a final concentration of 0.5% and 1.5%, respectively. Subsequently, the tissue samples were rapidly dehydrated by passing the specimen through an ascending series of ethanol solutions and then transferred into a 1:1 mixture of propylene oxide and epoxy araldite. Initially, semi-thin sections (1 µm) were cut using a microtome (0.5 µm thickness), stained with toluidine blue, and viewed under a light microscope to select suitable areas for electron microscopy. Furthermore, ultrathin sections (60 to 100 nm) were cut using a glass knife in an LKB microtome and stained with uranyl acetate and then by lead citrate (Hayat, 2012). Thereafter, sections were examined under a transmission electron microscope (TEM; JEOL 100cx, Peabody, MA, USA) operating at 80 kV (El Okle et al., 2016).

2.6 TUNEL assay

Liver cell apoptosis was analyzed using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) method described in a previous study (Wang et al., 2021a). The TUNEL assay was determined by using TUNEL Assay Kit (Abcam, Cambridge, United States) according to the manufacturer’s instructions. Briefly, the paraffin-embedded liver sections were
deparaffinized with xylene, hydrated with gradient concentrations of alcohol, and covered with proteinase K. Slides were incubated with terminal deoxynucleotidyl transferase and biotinylated nucleotides and then treated with salinesodium citrate buffer, 6% hydrogen peroxide, streptavidin–HRP conjugate, and DAB substrate solution. Finally, the slides were counterstained in hematoxylin solution. Images were captured using an Olympus microsystem (Tokyo, Japan).

2.7. Determination of ST colonization

ST colonies were detected in the ileum, colon, liver, spleen, and feces, as previously described (Taub et al., 2012). Briefly, samples were collected aseptically and homogenized in PBS containing 0.1% Triton X-100. Serial dilutions of organ homogenates were coated on Salmonella-Shigella (SS) agar plates (Guangdong Huankai Biological Co., Ltd.). CFUs was enumerated by visual micro-colony counting.

2.8 Detection of NO

Nitric oxide (NO) generation was assessed using the Griess method, as previously described (Mao et al., 2015). Briefly, samples were mixed with 100 µL of 1% sulfanilamide, 0.1% N-(1-naphthyl-) ethylenediamine dihydrochloride, and 2.5% phosphoric acid. Absorbance was measured at 550 nm within 5 min (Molecular Devices, San Jose, CA, USA), and the NO concentration was calculated using a standard curve created after performing serial dilutions of sodium nitrite.

2.9 Detection of MPO and caspase-1 secretion

Myeloperoxidase (MPO) activity in the colon and ileum and caspase-1 activity in the liver were determined using assay kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer’s instructions. Briefly, samples were added to wells, while assay buffer was added to standard wells. Reaction mix was then added to sample wells and incubated for 30 min to 2 h. Stop mix was added and incubated for an additional 10 min, after which TNB reagent/standard was added to all wells and incubated for 5–10 min. Plates were then analyzed with a microplate reader.

2.10 ELISA Detection

Tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-12p70, IL-6, and IL-10 levels in the culture supernatants, secretory immunoglobulin A (sIgA) in tissue homogenates, and IgG levels in plasma were measured using a sandwich ELISA kit (eBioscience, San Diego, CA, USA) as per manufacturer’s instructions.

2.11 Total RNA extraction and Quantitative Real-Time PCR

Total RNA was extracted from intestinal mucosal and MLN tissue samples using the RNAiso Plus Kit (Takara, Dalian, China) according to the manufacturer’s instructions. Complementary DNA was synthesized from 1 µg of total RNA using M-MLV reverse transcriptase (TaKaRa). Thereafter, transcriptional changes were detected using quantitative polymerase chain reaction performed (qRT-PCR) using SYBR Green Premix Ex Taq (TaKaRa) and the ABI 7500 Fast Real-Time PCR system (Applied
Biosystems, Carlsbad, CA, USA). The thermocycling protocol was as follows: 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing/extension at 60°C for 34 s. Melting curve analysis was performed to monitor the purity of the PCR product. The primer sequences are listed in Table S1. The $2^{-\Delta \Delta Ct}$ method was used to estimate mRNA abundance (where $\Delta Ct = Ct[\text{target}] - Ct[\text{reference}]$, and $\Delta \Delta Ct = \Delta Ct[\text{treatment}] - \Delta Ct[\text{control}]$). Relative gene expression levels were normalized against those of the eukaryotic $\beta$-actin gene.

2.12 DNA extraction and Illumina MiSeq

Microbial genomic DNA was extracted from fecal samples using the TIANamp Stool DNA kit (DP328; TIANGEN Biotech, Beijing, China) according to the manufacturer's recommendations. The V3-V4 hypervariable region of 16S rRNA was amplified from microbial genomic DNA, harvested from fecal samples ($n = 3$) using PCR (forward primer: 5′-CCTACGGGNGGCWGCAG-3′; reverse primer: 5′-GACTACHVGGGTATCTAATCC-3′). The volume of the reaction mixture was 20 µL; this contained 1× reaction buffer, 2 mM Mg$^{2+}$, 0.2 mM dNTP, 0.1 µM of each primer, 1 U HotStarTaq polymerase (cat. #203203; QIAGEN, Germantown, MD, USA), and 2 µL DNA template. The PCR program was set as follows: 94°C for 2 min; followed by 30 cycles of 94°C for 20 s, 52°C for 40 s, and 72°C for 1 min; and 72°C for 2 min; and PCR product storage at 4°C. The PCR system, used to add specific sequence tags, had a total volume of 20 µL and contained 1× reaction buffer (Q5TM; New England Biolabs (NEB), Ipswich, MA, USA), 0.3 mM dNTP, 0.25 M of each primer, 1 U Q5TM DNA polymerase (NEB), and 1 µL of diluted template. The PCR conditions were as follows: 98°C for 30 s; followed by 30 cycles at 94°C for 10 s, 65°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. The PCR product was excised after separation in 1.5% agarose gel, purified using the QIAquick Gel Extraction Kit (cat. #28706; QIAGEN), and quantified using a UV-Vis spectrophotometer (ND1000; NanoDrop Technologies, Wilmington, DE, USA). Library construction and Illumina MiSeq sequencing were performed at Hangzhou G-Bio Biotech Co., Ltd. (Hangzhou, China). Furthermore, alpha diversity (observed OTUs and PD_whole_tree) and beta diversity were analyzed based on a subsample of a minimum number of sequences (12,722) by QIIME software (Hu et al., 2018). The co-occurrence networks of microbial communities in different treatments were constructed based on significant correlations (Spearman’s R > 0.6 and FDR-adjusted P < 0.05) (Jiao et al., 2016) and were visualized by gephiTM software (https://gephi.org/).

2.13 Statistical analysis

Statistical analyses were performed by applying the two-tailed Student’s $t$-test and one way ANOVA using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA). Graphs were generated using GraphPad Prism version 6.00 for Windows (GraphPad Software, Inc., CA, USA). $P$ values < 0.05 were considered statistically significant.

3 Results
3.1 GOD suppress weight loss and mortality in ST-infected mice

No significant difference in body weight was observed among three groups (CK, GOD, and GM; \( p > 0.05 \)) during the experimental period (Fig. 1A). However, ST infection decreased the body weight of mice by 13.30% compared to the CK group \( (p < 0.01) \), which was suppressed by GOD pretreatment (the body weight was 13.94% higher than that in the ST group; \( p < 0.01 \)); similar results were obtained by GM pretreatment \( (p > 0.05; \) Fig. 1B). Moreover, GOD or GM pretreatment effectively enhanced the survival rate of ST-infected mice compared to the ST group (Fig. 1C). These results suggest that pretreatment with GOD or GM could effectively suppress weight loss and mortality in ST-infected mice.

3.2 GOD inhibit the colonization and translocation of ST in mice

The colonization and translocation of ST in the intestines of mice directly determines ST survival and pathogenicity. Compared to the ST group, GOD pretreatment inhibited ST colonization in the ileum and colon as well as its translocation to the spleen. Accordingly, the number of ST colonies \( (\log_{10} \text{CFU/g tissue}) \) decreased by 28.09% \( (p < 0.01) \), 28.85% \( (p < 0.01) \), and 21.48% \( (p < 0.05) \), respectively. Similar results were observed for GM pretreatment (Fig. 1D). The above results indicate that GOD pretreatment could inhibit the colonization and translocation of ST in mice.

3.3 GOD decrease organ coefficients increase colon length in ST-infected mice

The coefficients of the spleen, liver and colon length of normal and ST-infected mice are presented in Table 1. Compared to the CK group, the coefficient of the liver in normal mice significantly increased in the GM group \( (p < 0.05) \). However, ST infection significantly increased the liver and spleen indices of mice by 33.28% \( (p < 0.05) \) and 71.16% \( (p < 0.01) \), respectively, compared to those of the CK group, whereas GOD pretreatment decreased liver and spleen indices by 23.61% \( (p < 0.05) \) and 36.54% \( (p < 0.05) \), respectively compared to those of the ST group. Similar results were observed in the GM pretreatment group. The length of the colon in the control group was 8.08 cm, and no significant difference was observed in the average colon length between the CK and GOD groups. As expected, the length of colon markedly decreased in the ST group, while GOD and GM pretreatment increased colon length by 24.76% \( (p < 0.05) \) and 25.56% \( (p < 0.05) \), respectively, compared to the ST group.
Table 1
Effects of GOD on organ coefficients.

<table>
<thead>
<tr>
<th>Items</th>
<th>CK</th>
<th>GOD</th>
<th>GM</th>
<th>ST</th>
<th>GOD + ST</th>
<th>GM + ST</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen index (mg/g)</td>
<td>2.67b</td>
<td>2.74b</td>
<td>2.94b</td>
<td>4.57a</td>
<td>2.90b</td>
<td>2.76b</td>
<td>0.103</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver index (mg/g)</td>
<td>42.52d</td>
<td>45.51cd</td>
<td>47.42c</td>
<td>56.67a</td>
<td>43.29d</td>
<td>51.08b</td>
<td>1.108</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Colon length (cm)</td>
<td>8.08a</td>
<td>7.88a</td>
<td>8.06b</td>
<td>6.30b</td>
<td>7.86a</td>
<td>7.91a</td>
<td>0.391</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

a-d In the same row, values with different superscript letters mean significant difference (p < 0.05). Data are shown as the means of six mice.

3.4 GOD pretreatment could relieve intestinal mucosal injury induced by ST infection

H&E staining of tissue samples collected from normal mice (from the CK, GOD, and GM groups) exhibited clear and complete gland structures (ordered intestinal villi) in the ileal mucosa (Fig. 2A). Compared to the CK group, ST infection resulted in fragmentation of the ileal mucosa with sparse, shortened villi and sparsely distributed crypts (as indicated by the black arrow). However, pretreatment with GOD induced a dense arrangement of villi and deep glandular crypts. Moreover, normal mice in the GOD group exhibited complete and dense ileal microvilli (fully formed and closely arranged structures) and longer tight junctions. Similar results were observed with GM pretreatment (Fig. 2A). Not surprisingly, ileal micro-villi were severely damaged by ST infection, which was not observed in mice with GOD or GM pretreatment (Fig. 2B). Moreover, both GOD and GM groups exhibited a higher villi height and villi height/crypt depth ratio than that of the CK group. After ST infection, both the villi height and the ratio of villi height/crypt depth in the ST group markedly decreased compared to the control group, while GOD and GM pretreatment effectively alleviated this phenomenon (Fig. 2C). Therefore, GOD pretreatment could relieve intestinal mucosal injury induced by ST infection.

GOD significantly increased the mRNA expression of Zo1, claudin 1 (Cldn), and occludin (Ocln) in the colon of normal mice compared to the CK group (p < 0.01) and GM group (p < 0.05). ST infection significantly decreased the mRNA expression of Zo1 (p < 0.01), Cldn (p < 0.01), and Ocln (p < 0.05) compared to that in the CK group. However, the expression levels increased by 6.4-fold (p < 0.01), 15.9-fold (p < 0.01), and 3.1-fold (p < 0.05), respectively, in the GOD pretreatment group compared to ST group. Additionally, CLDN expression in the GOD pretreatment group increased by 72.52% compared to GM pretreatment group (p < 0.05; Fig. 2D).

3.5 GOD pretreatment could relieve apoptosis in liver of ST-infected mice
Figure 3A presents the representative H&E staining sections of liver, and images of mouse liver TUNEL-stained for the assessment of apoptosis. In the three normal mouse groups (CK, GOD, and GM groups) liver cells exhibited normal architecture. ST infection increased vacuolation of cells in the liver, and the cells were shrunken, and their cytoskeletal structure was disordered. In addition, compared with the ST group, the number of liver red blood cells in the COD and GM pretreatment groups decreased significantly. No obvious apoptosis was observed in the liver of normal mice, however, numerous apoptotic cells were increased in the hepatic foci of the ST infection group, which significantly decreased by pretreated with GOD or GM.

The above results are consistent with the caspase-1 activity results in the liver (Fig. 3B). Briefly, no significant differences in the caspase-1 activity were observed among the three normal mouse groups (CK, GOD, and GM groups). However, ST infection increased the activity of caspase-1 by 82.34% ($p < 0.01$) compared to the CK group, which decreased by 42.38% ($p < 0.01$) by GOD pretreatment. Similar results were observed in the GM pretreatment group. These results indicate that pretreatment with GOD exerted a superior anti-apoptosis effect.

### 3.6 GOD pretreatment could inhibit inflammatory responses by regulating cytokine secretion in ST-infected mice

As shown in Fig. 4A-E, GOD increased the secretion of the pro-inflammatory cytokine IFN-γ by 66.22% ($p < 0.01$) and decreased the secretion of IL-12p70 and TNF-α by 25.38% ($p < 0.05$) and 26.58% ($p < 0.05$), respectively, in the colonic mucosa of normal mice. Compared to the CK group, ST infection increased the secretion of IL-6 (29.07%, $p < 0.05$), IL-12p70 (25.78%, $p < 0.05$), TNF-α (56.77%, $P < 0.05$), and IFN-γ (149.20%, $p < 0.01$). However, pretreatment with GOD decreased the secretion of IL-6 (63.29%, $p < 0.01$), IL-12p70 (62.32%, $p < 0.01$), TNF-α (72.98%, $p < 0.01$), and IFN-γ (44.23%, $p < 0.05$) compared to the ST group. Notably, GOD pretreatment also increased the secretion of IL-10 by 23.53% ($p < 0.01$). Hence, GOD pretreatment inhibited intestinal inflammatory response by increasing IL-10 levels and decreasing the levels of pro-inflammatory cytokines (IL-6, IL-12p70, TNF-α, and IFN-γ) in ST-infected mice.

Cytokines in the gut enter the blood and, therefore, play an important role in protecting the gut from ST infection. GOD increased the IL-10 level by 30.63% ($p < 0.01$), while decreasing that of IL-12p70 and TNF-α by 38.86% ($p < 0.01$) and 19.67% ($p < 0.05$), respectively, in the serum of normal mice (Fig. 5A-E). ST infection increased the levels of inflammatory cytokines (IL-6, IL-12p70, TNF-α, and IFN-γ) and decreased IL-10 compared to the CK group. However, GOD pretreatment significantly suppressed the production of IL-6 ($p < 0.01$), TNF-α ($p < 0.05$), and IFN-γ ($p < 0.01$) and promoted the secretion of IL-10, which was similar to the results obtained in the colonic mucosa. These results suggest that GOD pretreatment could inhibit excessive inflammatory responses by regulating cytokine secretion in ST-infected mice.

### 3.7 GOD pretreatment could inhibit the excessive secretion of sIgA, NO, and MPO in ST-infected mice.
The secretion of sIgA, NO and MPO in the ileum and colon are shown in Fig. 6A-F. In normal mice, GOD significantly increased the sIgA level in the colonic mucosa, ST increased the production of sIgA in both ileal and colonic mucosa by 1.66-fold ($p < 0.01$) and 2.12-fold ($p < 0.05$), respectively. However, GOD pretreatment significantly suppressed the production of sIgA ($p < 0.01$) in the intestinal mucosa. No significant differences were observed between the GOD and GM pretreatment groups ($p > 0.05$). These findings suggest that pretreatment with GOD could effectively inhibit the excessive secretion of sIgA in ST-infected mice.

Compared to the CK group, ST increased the production of NO and MPO by 61.87% ($p < 0.01$) and 177.78% ($p < 0.01$), respectively, in the ileal mucosa, and by 198.95% ($p < 0.01$) and 195.24% ($p < 0.05$), respectively, in the colonic mucosa. However, GOD pretreatment significantly suppressed the production of MPO ($p < 0.01$) in the ileum and colon. No significant differences were observed between the GOD and GM pretreatment groups ($p > 0.05$). These findings suggest that pretreatment with GOD could effectively inhibit the excessive secretion of NO, and MPO in ST-infected mice.

### 3.8 GOD could improve the intestinal microbiota composition in mice

The cecal bacterial communities in healthy and ST-infected mice were examined using 16S rRNA sequencing. Alpha diversity parameters at the OTU level, including observed species, Chao1, PD_whole_tree, and Shannon indices, demonstrated that the richness and diversity of intestinal microbiota significantly decreased following GM pretreatment; however, this reduction was not observed in the GOD treatment or pretreatment group (Fig. 7A). Partial least squares discriminant analysis (PLS-DA) was performed to distinguish between different samples by establishing a model of the relationship between species abundance and sample category. Results revealed clear separation and distinction of the ST group from the other five groups (CK, GOD, GM, GOD + ST, and GM + ST; Fig. 7B).

Results from LEfSe analysis, representing the structure of the cecal microbiota and predominant bacteria, are shown in Fig. 8A. In normal mice, GOD treatment was associated with a significant increase in various taxa, namely, *Lactobacillales*, *Lactobacillaceae*, *Bacilli*, *Lactobacillus* and *Ruminococcus*. Meanwhile, ST infection markedly increased the relative abundance of *Clostridia*, *Firmicutes*, *Clostridiales*, *Lachnospiraceae*, *Coprococcus*, *Alphaproteobacteria*, *Bacteroidesacidifaciens* and *Roseburia*, whereas pretreatment with GOD significantly promoted the relative abundance of *Deltaproteobacteria*, *Desulfovibrionales*, *Proteobacteria*, *Campylobacterales*, *Epsilonproteobacteria*, *Helicobacteraceae*, *Anaerostipes*, *Rikenella*, *Tenericutes* and *Mollicutes*.

Differences in the intestinal bacterial composition was also assessed between groups (Fig. 8B-D). The results showed that ST infection significantly decreased the relative abundance of *Dehalobacterlum*, *Desulfovibrionaceae*, *Desulfovibrionales*, *Deltaproteobacteria*, *Lactobacillaceae*, *Lactobacillales*, *Lactobacillus*, *Proteobacteria*, *Bacilli*, *Rikenella* and *Odoribacter*, while increasing the relative abundance of *Bacteroidaceae*, *Bacteroides*, *Coprococcus* and *Bacteroides acidifaciens*. Moreover, GOD pretreatment significantly increased the relative abundance of *Lactobacillales*, *Bacilli*,...
Lactobacillaceae, Lactobacillus, Proteobacteria, Desulfovibrionaceae, Desulfovibrionales, Deltaproteobacteria, which were decreased by ST infection. Additionally, the relative abundance of Coproccoccus, Bacteroidaceae, Bacteroides, Alphaproteobacteria and RF32 were significantly increased in the GOD + ST group compared with the ST group. Compared to the ST group, GM pretreatment significantly decreased the relative abundance of Clostridiales, Clostridia, Firmicutes, Dehalobacteriaceae, Dehalobacterium and Lachnospiraceae, and markedly increased the relative abundance of Alistipes indistinctus, Peptostreptococcaceae, Clostridium, Bacteroidetes, Bacteroidales, Bacteroidia, Betaproteobacteria and Eubacterium.

To determine the co-occurrence patterns of microbes in the CK, ST, GOD + ST and GM + ST groups, four networks were constructed at the phylum level (Fig. 9 and Table S2). Network analysis showed that the average degree and graph density in the ST group was higher than the other groups (CK, GOD + ST and GM + ST), suggesting that ST infection increased the connection among bacterial species. Moreover, the modularity values of the co-occurrence networks in four groups were higher than 0.8, indicating a modular structure. The modularity in the ST group was lower than both GOD + ST and GM + ST groups; hence, the microbial network in the ST group did not readily form a “small world” compared to the other infected groups. Additionally, the negative correlation associated with the network in the ST group was lower than that in the GOD + ST and GM + ST groups, which may indicate an increased competitiveness within intestinal microbes.

4 Discussion

Glucose oxidase reportedly exhibits growth-promoting properties in animals, including ducks, broilers and pigs (Liu et al., 2020; Tang et al., 2016; Wu et al., 2019b), while also exerting non-toxic and low-residue antibacterial and antifungal effects (Wang et al., 2018; Wong et al., 2008). In the present study, although GOD supplementation did not markedly increase mouse growth performance, it effectively reduced the weight loss, mortality rate, pathogen colonization and translocation associated with ST infection. Thus, GOD pretreatment could inhibit ST infection and suppress the weight loss and mortality in ST-infected mice.

After infecting the host, Salmonella propagates rapidly and spreads through the bloodstream to invade the liver, spleen, heart, and other organs, causing pathological changes in animal internal organs, which then affect host physiological activities, and ultimately leads to animal death (Herrero-Fresno and Olsen, 2018; Troxell et al., 2015; Wu et al., 2018). The present study illustrated that the relative weight of both liver and spleen did not increase in the GOD group in normal mice, while these parameters increased significantly after ST infection. This symptom may be caused by ST pathological damage to the mice, and is consistent with the results of Wang et al., who reported that the relative weight of the spleen and liver in mice was markedly increased in ST-challenged groups (Wang et al., 2021b). The findings of the current study also indicated that orally administered GOD abrogated the higher liver and spleen index caused by ST infection, indicating that GOD pretreatment, similar to the GM pretreatment, exerted protective effects against tissue injury induced by Salmonella infection.
ST-induced intestinal injury is one of the most common characteristics of its infection. Its major pathological damage to the cell is architectural disorganization, resulting in reduced villi height (Zhang et al., 2020). The findings of the present study show that the villi of ileum were thickened with markedly reduced villi height following ST infection, indicating that the ST-induced intestinal injury model was successfully established. Thus, the height of the villi determines the surface area available for intestinal absorption and affects the growth and development of animals (Wilson et al., 2018). After ST infection, the height of villi and the villi height to crypt depth ratio decreased, indicating that the intestinal structure and its absorption function might be impaired (Li et al., 2021). GOD or GM pretreatment effectively alleviated these effects. These results confirm the findings of previous studies that GOD supplementation increase villi height in the jejunum of ducks (Liu et al., 2020). Hence, GOD pretreatment could alleviate the intestinal damage induced by ST-infected.

ST challenge can lead to impaired intestinal mucosal barrier function (Yu et al., 2018). The epithelial barrier is one of the most important components of the intestinal mucosal barrier to prevent the transmission of macromolecules (Martini et al., 2017). Epithelial cells form a continuous and complete physical barrier, with tight junctions (TJ) between each cell. The destruction of TJs results in increased permeability to luminal antigens and bacteria, and decreased mucosal barrier function. Several bacterial pathogens can alter TJ by altering the TJ-associated proteins (Zhang et al., 2019). Zhang et al., indicated that ST may destroy the structure and function of TJs, thereby destroying the integrity of the epithelial barrier (Zhang et al., 2012). Herein, the qRT-PCR results demonstrated that GOD or GM pretreatment enhanced intestinal TJs by up-regulating the transcription level of tight junction proteins ZO-1, occludin, and claudin-1.

The liver, which is constantly exposed to foreign material, owing to gut translocation and first-pass metabolism from the bloodstream, plays a key role in various physiological activities, such as nutrient metabolism, blood detoxification, and the immune response (Nguyen et al., 2014). Previous studies have shown that Salmonella infections are accompanied by damage to the liver, spleen and other organs (Fabrega and Vila, 2013; Song et al., 2020), as evidenced by inflammatory cell infiltration, severe congestion, and hepatocyte apoptosis due to the complex interaction between the intestine and liver through the gut-liver axis (Abatemarco et al., 2018; Wei et al., 2019). As expected, ST infection led to the liver cells becoming shrunken and severely congested. We also found that ST infection markedly increased apoptosis and the expression of the pro-inflammatory protein caspase-1, which were effectively inhibited by GOD pretreatment.

Inflammation is considered to be the basic mechanism of pathophysiology in intestinal injury (Ma et al., 2021). The relative increase in the abundance of certain inflammatory factors may weaken the expression of ZO-1, cause intestinal barrier dysfunction, and promote inflammation of the intestinal mucosa (Ogawa et al., 2018). NO is a highly soluble, reactive free radical as well as a potent intercellular and intracellular signaling molecule that acts as a pro-inflammatory agent (Beckman et al., 1990; Levy et al., 2005). Moreover, NO can increase the cellular expression of TNF-α and IL-1β, and enhance the ability of the cell to produce H₂O₂ (Lander et al., 1993; Magrinat et al., 1992). We found that ST infection
resulted in a dramatic increase in NO abundance in both the ileum and colon, as well as increased MPO activity, an indicator of an increase in the number of neutrophilic granulocytes. Interestingly, the adverse effects of ST infection were abrogated by GOD or GM pretreatment, indicating a reduction in tissue injury and neutrophil infiltration.

Increasing experimental evidence agrees with the viewpoint that the abnormal regulation of the mucosal immune system in response to infection by enteric bacteria leads to intestinal injury (Guarner 2005; Nozawa et al., 2004). Moreover, inflammation is also a key factor affecting the intestinal mucosal barrier (Huang et al., 2021). Cytokines IL-6, TNF-α, and IFN-γ are primarily derived from mononuclear phagocytes and other antigen-presenting cells, which play an important role in promoting cell infiltration and damage to resident tissues, characteristic of inflammation (Santamaria, 2003). In addition, IL-10 is a multicellular and multifunctional cytokine that is considered to be an inflammatory and immunosuppressive factor, that regulates cell growth and differentiation and participates in inflammation and immune responses (Huang et al., 2018). Therefore, the decreased levels of IL-6, IL-12, TNF-α, and IFN-γ, as well as the increased IL-10 levels induced by GOD pretreatment suggest its anti-inflammation response and protective effects on the mucosal barrier.

Ig is a specific active protein that is transformed into antibodies (IgG, IgA, IgM, IgE, and IgD) following antigen induction (Liu et al., 2020). sIgA, secreted by intestinal epithelial cells, is critical for preventing the damage induced by inflammatory bowel disease by inhibiting the attachment of pathogenic microorganisms to the gastrointestinal tract (Lammers et al., 2010). In the present study, GOD significantly enhanced the production of sIgA in normal mice. It should be noted that this also occurred in the ileum and colon after ST infection, which may be due to the adhesion of a large number of pathogenic bacteria, such as ST, in the intestine. Similar results also reported that GOD promotes serum Ig levels so as to enhance the ability to eliminate pathogenic microorganisms (Wu et al., 2019).

Previous studies have shown that certain adverse factors are associated with the application of antibiotics, particularly the disruption of intestinal bacterial diversity in antibiotic-supplemented groups (Liu et al., 2020). However, GOD supplementation helped overcome these shortcomings, which was in agreement with the findings of our current study on mice. The intestinal microbiota plays a vital role in host defense, as evidenced by its ability to regulate innate and acquired immunity at the local and systemic levels (Colliou et al., 2017). Most importantly, changes in microbial populations may be closely related to the degree of intestinal inflammation, which is also a characteristic of ST infection (Bronner et al., 2018). Throughout history, there have been reports of protection against ST infection via regulation of intestinal microbiota (Baba et al., 1991; Deriu et al., 2013). Herein, 16S rRNA sequencing indicated that GOD treatment or pretreatment had considerable regulatory effects on the intestinal microbiota; PL-SDA results further revealed that oral GOD administration restructured the flora of diseased intestines to be similar to that of the CK group.

*Ruminococcaceae* are primarily responsible for fermenting dietary fiber and other plant components, such as inulin and cellulose, to produce SCFAs, which can be used as energy by the host (Scheppach and
Weiler, 2004) and exhibit anti-inflammatory properties in the intestine (Kles and Chang, 2006). An abundance of Lactobacillaceae is associated with T cell counts in the peripheral blood, while Lactobacillus, specifically, can effectively enhance the innate and adaptive immunity of the host (Ma et al., 2017). Furthermore, Lactobacillus species are also promising probiotic candidates as they exhibit antimicrobial activities, which are attributed to the secretion of bacteriocins or the production of organic acids. In the present study, GOD markedly increased the relative abundance of Ruminococcus and Lactobacillaceae in the intestines of normal mice. Furthermore, the decreased relative abundance of Lactobacillales, Lactobacillaceae and Lactobacillus following ST infection were abrogated by GOD pretreatment. These findings indicate that GOD can regulate the abundance of specific intestinal microbiota to provide protection against ST infection, which may also account for why GOD pretreatment was found to effectively reduce ST colonization and translocation. Nevertheless, the differences induced in the diversity of mouse fecal microbiota following application of the six treatments requires further investigation, particularly with respect to the association between the specific characterization of GOD and the metabolic products of gastrointestinal tract microbiota.

5 Conclusions

In conclusion, orally administered GOD could inhibit the weight loss, ameliorate microbial dysbiosis and excessive inflammation caused by ST infection, and inhibit pathogen colonization and translocation, while promoting reproduction of beneficial bacteria and intestinal barrier function. Collectively, GOD administration may represent a safe strategy for the prevention and treatment of ST infection. Hence, these results provide a potential reference for the clinical application of GOD in the future.

Declarations

Acknowledgments

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Author contributions

Y.W. and Y.W. led the design and performance of the experiments. Y. W. and B.W. contributed to the analysis of the data, and the writing of the paper. Y.W., Y. Z. and Y.W. participated in the animal feeding trials. W.L, Y.W., X. Z and B.Y. participated in designing the experiments and editing the paper. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

All authors declare that they have no conflict of interest.
References


**Figures**

![Figure 1](image)

**Figure 1**

Effects of GOD on the body weight of normal and ST-infected mice. **(A)** Body weight was monitored every 3 days over 28 days. **(B)** Mice were infected orally with $4 \times 10^8$ CFU of *Salmonella*, except for the CK, GOD and GM-alone groups. The weight loss of mice at 72 h post-infection. **(C)** The survival rate of mice at 72 h post-infection. **(D)** The colonization of ST in ileum, colon, liver, spleen and feces were detected at 72 h post-infection. Significance was analyzed using a paired t-test for body weight ($n = 8$/group; **$P < 0.01$, *$P < 0.05$ were accepted as statistically significant; ns, not significant).
Figure 2

Effect of GOD on the intestinal structure of normal and ST-infected mice. (A) Representative H&E staining sections of ileum in normal and ST-infected mice. (B) Transmission electron microscopy (bottom, scale bars = 1.0µm) of ileum mucosal surface of mice. (C) Statistical analysis of the histological parameter ‘villus height and villus height:crypt depth ratio’ in the ileum. (D) Changes in the relative expression of ZO-1, CLDN and OCLN in the jejunum. Significance was analyzed using a paired t-test (n = 4/group; **P < 0.01, *P < 0.05 were accepted as statistically significant; ns, not significant).

Figure 3

Effects of GOD on the liver structure of normal and ST-infected mice. (A) Representative H&E staining sections of liver (left) and images of the mice liver stained for TUNEL (right). (B) Caspase-1 activity in
liver of normal and ST-infected mice. Significance was analyzed using a paired t-test (n = 4/group; **P < 0.01, *P < 0.05 were accepted as statistically significant; ns, not significant). The arrows indicate the vacuolation of cell or the blood cell. Scale bar, 80µm.

Figure 4

Effect of GOD on cytokine secretion in normal and ST-infected mice. Levels of IL-10 (A), IL-6 (B), TNF-α (C), IFN-γ (D) and IL-12 (E) secretion in colon. Significance was analyzed using a paired t-test (n = 8/group; **P < 0.01, *P < 0.05 were accepted as statistically significant; ns, not significant).
Figure 5

Effect of GOD on cytokine secretion in normal and ST-infected mice. Levels of IL-10 (A), IL-6 (B), TNF-α (C), IFN-γ (D) and IL-12 (E) secretion in plasma. Significance was analyzed using a paired t-test (n = 8/group; **P < 0.01, *P < 0.05 were accepted as statistically significant; ns, not significant).
Figure 6

Effects of GOD on sIgA, NO and MPO secretion in the mucosa of ileum (A, B and C) and colon (D, E and F) were quantified by ELISA. Significance was analyzed using a paired t-test (n = 8/group; **P < 0.01, *P < 0.05 were accepted as statistically significant; ns, not significant).

Figure 7

(A) Alpha diversity analysis of intestinal microbiota in mice; (B) Partial Least Squares Discriminant Analysis of gut microbiota at the operational taxonomic unit (OTU) level. Significant difference versus CK
Figure 8

(A) Compare of enriched taxa based on LEfSe analysis reveals significant differences of the microbial community between groups. Bacterial taxa with LDA score > 3 are selected as biomarker taxa [p: phylum level, c: class level, o: order level, f: family level, g: genus level]; (B, C and D) Comparison of the intestinal
bacteria among different treatments by statistical analysis of taxonomic and functional profiles (STAMP).

Figure 9

Co-occurrence networks of microbial communities at phylum level. A connection stands for a very strong (Spearman's R > 0.6) and significant (FDR-adjusted P < 0.05) correlation. The size of each node is
proportional to the relative abundance; the thickness of each connection between two nodes (edge) is proportional to the value of Spearman's correlation coefficients. Red lines represent significant positive correlations and green lines denote negative correlations.

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