Prevention and Curation of DSS-induced IBD in Mice With Bacillus Subtilis Fermented Milk via Inhibition of the Inflammatory Responses and Regulation of the Intestinal Flora

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Research

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Abstract

**Background:** The pathogenesis of inflammatory bowel disease (IBD) might be related to the local inflammatory damage and the dysbacteriosis of intestinal flora. Probiotics can regulate the intestinal flora and ameliorate IBD. The probiotic *Bacillus subtilis* strain *B. subtilis* JNFE0126 was used as the starter of fermented milk. However, the therapeutic effects of *B. subtilis* fermented milk on IBD remains to be explored.

**Methods** The therapeutic effect of the *B. subtilis* fermented milk on DSS-induced IBD model mice was evaluated. The disease activity index (DAI) and the pathological features of small intestinal and colonic mucosa were examined. For exploring the action mechanism of *B. subtilis*, immunohistochemical staining and western-blotting were used to analyse the expression of the pro-inflammatory/anti-inflammatory cytokines, the proliferation of the intestinal stem cells, and the reconstruction of the mucosa barrier. The alteration of gut microbiota was investigated by taxonomic analysis.

**Results:** The DAI of IBD was significantly decreased through oral administration of *B. subtilis* (JNFE0126) fermented milk, and the intestinal mucosa injury was attenuated. Moreover, *B. subtilis* could reduce the inflammatory response of the intestinal mucosa, induce proliferation of the intestinal stem cell, and promote reconstruction of the mucosal barrier. Furthermore, *B. subtilis* could rebalance the intestinal flora, increasing the abundance of *Bacillus, Alistipes* and *Lactobacillus*, while decreasing the abundance of *Escherichia* and *Bacteroides*.

**Conclusion:** Oral administration of the *B. subtilis* fermented milk could alleviate DSS-induced IBD via inhibition of inflammatory response, promotion of the mucosal barrier reconstruction and regulation of the intestinal flora.

Introduction

IBD is a chronic inflammatory disease of intestines, which has emerged as global diseases. The robust epidemiologic and experimental studies have defined the role of the lifestyle and microbiome on the pathogenesis of this disease. The pathogenesis of IBD is associated with intestinal flora imbalance and over expression of pro-inflammatory cytokines due to autoimmune response, which results in apoptosis of epithelial cells and formation of local ulcer. Pharmacotherapy of IBD includes the use of aminosalicylates, corticosteroids, 6-mercaptopurine, and other immunosuppressive agents. Recently, the anti-TNF antibodies have become a novel strategy. However, the fore-mentioned therapies, with varied degree, have side-effects and limitations. In comparison with the traditional therapy and monotherapy, probiotic/dietary therapy and combinations of some therapeutic approaches might possess better patient acceptability and therapeutic effect in the prevention and curation of IBD.

Recent reports revealed that the alteration of gut microbiota was associated with IBD, including the change of relative abundance and the decreased diversity of the microbiota. This reduction in richness of
the microbiota was seen for Firmicutes, Actinobacteria, and Bacteroidetes, Collinsella, Lactobacillus, and Bacillus.\textsuperscript{11–13} To restore flora balance in the IBD, some probiotic products have been applied to regulate the intestinal flora for treatment of this disease in recent reports.\textsuperscript{14–19} Fermented foods, which contained edible microorganisms, have been investigated as dietary supplements for prevention and treatment of IBD. Fermented barley and soybean showed the protective function against IBD through the effects on the inflammatory reaction, tight junction protein expression, and gut microbiota composition in animals;\textsuperscript{20} Saraiva et al. reported that milk fermented with a 15-lipoxygenase-1-producing Lactococcus lactis alleviated symptoms of colitis in a murine model.\textsuperscript{21}

\textit{B. subtilis} is a probiotic found in the Japanese traditional food natto. Recent clinical researches reported that oral intake of \textit{B. subtilis} was effective in preventing pediatric diarrhea and reducing the duration of diarrhea.\textsuperscript{22} \textit{B. subtilis} can secret some bioactive substances beneficial to human health and has been applied as a probiotic in humans.\textsuperscript{23} Yan et al. reported that surfactin A from \textit{B. subtilis} could inhibit the inflammation and promote the wound healing.\textsuperscript{24} Johnson et al. reported that Peptidoglycan-Associated Cyclic Lipopeptide (a surfactin) from \textit{B. subtilis} could disrupt CoV virion integrity in the HUH7 cells and reduce infection >10,000-fold, and its viricidal activity extended broadly to enveloped viruses, including SARS-CoV, MERS-CoV, influenza, Ebola, Zika, Nipah, chikungunya, Una, Mayaro, Dugbe, and Crimean-Congo hemorrhagic fever viruses.\textsuperscript{25} It was also reported that Poly-γ-glutamin secreted from \textit{B. subtilis} could attenuate the symptoms and histological features of IBD and reduce inflammation in an animal model of colitis.\textsuperscript{26} Foligné et al. reported that spores from \textit{B. subtilis} strain PB6 possessed the anti-inflammatory effects on the TNBS-induced colitis in mice.\textsuperscript{27} In addition, some functional factors in milk, such as conjugated linoleic acid (CLA) and milk polar lipids (MPLs) have been reported to possess function of anti-inflammation. CLA could ameliorate DSS-induced colitis in mice model,\textsuperscript{28} and MPLs could ameliorate necrotizing enterocolitis (NEC) in rat model by inhibiting the inflammatory response.\textsuperscript{29} Lorea et al. reported that the yogurt contained probiotics possessed anti-inflammatory effects in IBD patients.\textsuperscript{30} These reports prompt that the \textit{B. subtilis} fermented milk might be applied as a functional food for prevention and curation of IBD in the future.

IBD includes Ulcerative Colitis (UC) and Crohn's Disease (CD). CD has more serious symptoms due to the extensive inflammatory injury of both the small intestinal and colonic mucosa, compared to UC which showed limited pathological area of ulcers in the colon. For investigation of the pathogenesis and therapeutic mechanisms of drugs for IBD, some IBD animal model was applied for this research. Dextran Sulphate Sodium Salt (DSS) can corrode the mucosa and epithelium of the digestive tract and is widely used to establish animal models of IBD by oral intake of this chemical agent. In most present reports on DSS-induced IBD models, only the colonic damage was investigated, and the pathological changes in the small intestine were ignored. However, it was reported by Elshenkh et al. that the mucosal injury of the DSS-induced IBD existed extensively in the small intestine and the colon, which was similar to the pathological features of CD.\textsuperscript{31}
In the present study, the aim of this research was to identify the biological effect of orally administered \textit{B. subtilis} fermented milk based on the DSS-induced IBD animal experiment. For evaluation of the prevention and therapeutic effect of the \textit{B. subtilis} on DSS-induced IBD, the disease activity index (DAI) and the histological/pathological features of small intestinal and colonic mucosa were observed. Furtherly, the expression of inflammatory cytokines, the reconstruction of the epithelium barrier, and the alteration of intestinal flora were investigated to provide the underlying mechanism by which the \textit{B. subtilis} fermented milk alleviates IBD.

**Results**

The appearance of the \textit{B. subtilis} fermented milk and the microscope observation of the Gram staining sections

After 8 h of fermentation, the \textit{B. subtilis} fermented milk formed a firm curd with yellowish-white color, which showed no whey separation. After stirring, the \textit{B. subtilis} fermented milk became viscous fluid. This fermented milk possessed the sensory properties and overall acceptability similar to traditional fermented milk product (data not shown). The gram positive \textit{bacillus} in this fermented milk could be observed after gram staining, and the bacterial concentration was $6 \times 10^8$ CFU/ml.

**Effect of the \textit{B. subtilis} fermented milk on the disease severity of the DSS-induced IBD**

For evaluation of the disease severity of DSS-induced IBD, the DAI scoring was performed and the length of the colon was measured. As shown in Fig. 1A, during the second 7d period (the active phase of IBD), the DAI of the DSS group, the DSS + milk group and the DSS + \textit{B. subtilis} fermented milk group increased continuously as the DSS intake extended. However, DAI in DSS + \textit{B. subtilis} fermented milk group was lower than those of the DSS group and the DSS + milk group. In the third 7d period (the recovery phase of IBD), after termination of DSS intake, the recovery of DAI with varying degrees was observed in the DSS group, the DSS + milk group, and the DSS + \textit{B. subtilis} fermented milk group. However, in the DSS + \textit{B. subtilis} fermented milk group, the DAI index recovered faster and was significantly lower than those of the DSS group and DSS + milk group. It was suggested that the \textit{B. subtilis} fermented milk could effectively lessen the symptoms of the DSS induced IBD in the active phase. Moreover, after termination of DSS irritation, the recovery from the DSS induced injury was also promoted by oral intake of the \textit{B. subtilis} fermented milk. These results suggested that oral intake of DSS could induce injury of the intestines, however, oral administration of the \textit{B. subtilis} fermented milk could significantly lighten the symptoms of DSS-induced IBD. As shown in Fig. 1B and C, at 7d after the termination of DSS intake, the colons in the DSS group and the DSS + milk group were shortened significantly compared to the normal group and \textit{B. subtilis} fermented milk group. However, in the DSS + \textit{B. subtilis} fermented milk group, the colons were shortened only slightly and were significantly longer than those of the DSS group and the DSS + milk group. It was suggested that the \textit{B. subtilis} fermented milk could reduce the injury of the colons induced by DSS. In this experiment, the \textit{B. subtilis} fermented milk group showed no obvious difference
from the normal group, and the DSS + milk group showed no obvious difference from the DSS group, so that the *B. subtilis* fermented milk and the DSS + milk group were dismissed in the following sections about pathological observation and cell cytokine detection.

Effect of the *B. subtilis* fermented milk on the pathological changes in the small intestine and the colon

The tissue sections of the small intestines and the colons in the different groups were stained with eosin-hematoxylin and alcian blue. The pathological changes of the intestinal mucosa in the active phase and recovery phase of IBD were observed and compared.

**Effect of the *B. subtilis* fermented milk on the pathological changes in the small intestine**

As shown in Fig. 1D1, E1, for normal mice, the small intestinal mucosa was integrate, with the intestinal villi and the crypts arranged well. The epithelial cells covering the villi were closely arranged with no defects. The goblet cells with abundant mucus (blue) were observed in the villi and crypts. For the DSS group, at 7d after oral intake of DSS (the active phase of IBD), villi disintegration, epithelial exfoliation and glandular structure destruction were observed in the small intestinal mucosa due to formation of inflammatory ulcer induced by DSS (Fig. 1D2, E2); At 7d after the termination of DSS intake (the recovery phase of IBD), the damage in the small intestinal mucosa recovered partially, but the villus structure was still disordered, and the epithelium was not intact (Fig. 1F2, G2). However, for the DSS + *B. subtilis* fermented milk group, after 7d of DSS intake (the active phase of IBD, Fig. 1D3, E3), the damage of small intestinal mucosa was slight. Local superficial ulcer and partial abscission of the epithelial cells were observed. The crypt structure was almost intact and abundant mucus was observed in the goblet cells; At 7d after termination of DSS intake (the recovery phase of IBD, Figure F3, G3), the ulcer was repaired by regenerated epithelium and the intact epithelial barrier covering the mucosa was already reconstructed. The regenerative villus arrangement was still relatively scattered compared to the villus in the normal small intestine, but the recovery of the intestinal epithelium in the DSS + *B. subtilis* fermented milk was obviously better than that of the DSS group. These results suggested that oral intake of *B. subtilis* fermented milk could not only prevent the DSS induced damage of the small intestinal mucosa in the active phase of the IBD, but also promote repairing of the injury in the recovery phase of IBD.

**Effect of the *B. subtilis* fermented milk on the pathological changes in the colon**

In the normal colon (Fig. 1H1, I1), the epithelial cells were closely arranged with no local defects. The goblet cells with abundant mucus (blue) were observed in the glands. For the DSS group, after 7d administration of DSS (Fig. 1H2, I2), the damage of the colonic mucosa was more severe than that of the small intestine shown in Fig. 1D2, E2. The bleeding and deep ulcer were observed obviously. The epithelial cells and goblet cells were mostly destroyed. There was only scattered distribution of crypt epithelial cells and goblet cells in the ulcer. At 7d after termination of DSS intake (Fig. 1J2, K2), local epithelium was lost completely, and the scar was formed due to the hyperplasia of inflammatory tissue.
The scar tissue was exposed to the lumen without epithelial cell covering. However, for the DSS + *B. subtilis* fermented milk group, the damage in the colonic mucosa was slighter than that of the DSS group. The surviving glands and goblet cells were observed (Fig. 1H3), and there was abundant mucus in the goblet cells (Fig. 1I3); At 7d after termination of DSS intake, the damage in the colonic mucosa was obviously recovered, and there were regenerative epithelial and glands in the colonic mucosa (Fig. 1J3, K3). These results suggested that oral administration of the *B. subtilis* fermented milk could significantly reduce the DSS-induced damage of colonic mucosa in the active phase of the IBD, and promote the regeneration of the epithelial cells and glands of the injured colonic mucosa in the recovery phase of IBD.

Effect of the *B. subtilis* fermented milk on the neutrophil infiltration and expression of the inflammatory/anti-inflammatory cytokines

For evaluation of the inflammatory neutrophil infiltration in the intestinal mucosa, immunohistochemistry staining of neutrophil specific MPO was used to show the neutrophils. As shown in Fig. 2A1, in the normal small intestinal mucosa, few MPO staining positive neutrophils were observed. For the DSS group (Fig. 2A2), massive accumulative the MPO staining positive neutrophils were observed beneath the mucosa epithelium at 7d after termination of intake of DSS; For the DSS + *B. subtilis* fermented milk group (Fig. 2A3), however, only limited neutrophils could be observed in the small intestinal mucosa at 7d after termination of intake of DSS. Compared to the small intestine (Fig. 2A), the similar difference, between the DSS group and the DSS + *B. subtilis* fermented milk group, was observed in the colonic mucosa (Fig. 2B). In the normal colonic mucosa (Fig. 2B1), few neutrophils were observed. For the DSS group (Fig. 2B2), at 7d after the termination of DSS administration, the colonic epithelium and the glands disappeared, and the ulcer was locally replaced by scars and massive accumulative MPO positive neutrophils were observed in the scars. However, in the DSS + *B. subtilis* fermented milk group (Fig. 2B3), only limited neutrophils could be observed in the regenerative colonic mucosa. These results suggested that oral administration of the *B. subtilis* fermented milk could significantly reduce inflammatory severity of DSS-induced IBD. To explore the anti-inflammatory mechanisms of the *B. subtilis* fermented milk, the pro-inflammatory cytokine TNF and anti-inflammatory cytokine IL-10 were stained with IHC. In normal small intestinal mucosa (Fig. 2C1), the villus epithelium was integrate with low TNF expression. For the DSS group (Fig. 2C2), at 7d after the termination of DSS administration, the villus structure is not integrate, with high TNF expression. For the DSS + *B. subtilis* fermented milk group (Fig. 2C3), the villus and the glands were almost integrate, the relative expression level of TNF was significantly lower than that of the DSS group. It was suggested that administration of the *B. subtilis* fermented milk could significantly reduce TNF expression in the small intestine. Compared to the small intestine (Fig. 2C), the similar difference, between the DSS group and the DSS + *B. subtilis* fermented milk group, was also observed in the colon (Fig. 2D). For the normal colonic mucosa (Fig. 2D1), the epithelium and the colonic glands were integrate with low TNF expression; For the DSS group (Fig. 2D2), at 7d after the termination of DSS administration, the epithelium structure and the glands were destroyed and replaced by the inflammatory scars, which over-expressed TNF. For the DSS + *B. subtilis* fermented milk group (Fig. 2D3), the colonic epithelium and the glands were almost integrate, the relative expression level of TNF was significantly lower than that of the DSS group. As shown in Fig. 4E and F, in the normal small intestinal
mucosa (Fig. 2E1) and colonic mucosa (Fig. 2F1), the middle immunohistochemistry staining of IL-10 was mainly located in the small intestinal crypts and colonic glands. For the DSS group (Fig. 2E2, 22), IL-10 staining was observed in the residual small intestinal epithelium and the crypts (Fig. 2E2). In inflammatory scar of colonic mucosa, there were a few inflammatory cells with positive IL-10 staining (Fig. 2F2); For the DSS + *B. subtilis* fermented milk group (Fig. 2E3, F3), the dark brown staining in the regenerative epithelium of the small intestinal mucosa (Fig. 2E3) and colonic mucosa (Fig. 2F3) represented high-level expression of IL-10. For quantification analysis of the expressions of MPO, TNF and IL-10, the western blotting was used to show the relative expression levels of these proteins. Figure 2G and H showed that the relative expression level of MPO, TNF and IL-10 in the DSS group was significantly higher than that of the normal group. The expression of MPO and TNF in the DSS + *B. subtilis* fermented milk group was significantly lower than that of the DSS group, while the expression of IL-10 in the DSS + *B. subtilis* fermented milk group was significantly higher than that of the DSS group. These results suggested that the *B. subtilis* fermented milk could inhibit the expression of pro-inflammatory cytokine TNF and promote the expression of anti-inflammatory cytokine IL-10 in the mucosa of the small intestine and colon of the DSS-induced IBD model animals.

**Effect of the B. subtilis fermented milk on the expression of Lgr5, CDX2 and Mucin2**

The Lgr5 is the marker of the intestinal stem cells, which could differentiate to the epithelial cells and goblet cells of the mucosa in the small intestine and the colon. In the normal small intestinal mucosa, the Lgr5 positive stem cells were observed in the crypts (Fig. 3A1). For the DSS group, at 7d after termination of DSS intake, the Lgr5 positive stem cells reduced significantly and were observed in the bottom of crypts in the small intestinal mucosa (Fig. 3A2). For the DSS + *B. subtilis* fermented milk group, at 7d after termination of DSS intake, more Lgr5 positive stem cells were observed in the crypts of the small intestinal mucosa (Fig. 3A3). Meanwhile, in the normal colonic mucosa, the Lgr5 positive stem cells located deep in the colonic glands (Fig. 3B1). For the DSS group, at 7d after termination of DSS intake, the Lgr5 positive stem cells almost disappeared due to the formation of inflammatory ulcer and scar in the colonic mucosa (Fig. 3B2). For the DSS + *B. subtilis* fermented milk group, at 7d after termination of DSS intake, a lot of Lgr5 positive stem cells were observed in the colonic mucosa (Fig. 3B3). For evaluation of the ability of the Lgr5 positive stem cells to differentiate into the epithelial cells and the goblet cells, the epithelium cell marker CDX-2 and the goblet cell marker Mucin2 were stained with IHC. In the normal small intestinal mucosa, CDX-2 positive epithelial cells covered the villi and crypts and formed an integrated epithelial barrier (Fig. 3C1). For the DSS group, the villi and the glands were scattered due to the inflammatory damage, and few CDX-2 positive epithelial cells were observed in the residual villi and crypts (Fig. 3C2). For the DSS + *B. subtilis* fermented milk group (Fig. 3C3), more regenerative villi and crypts were observed and there were more CDX-2 positive epithelial cells covering the villi and crypts in comparison with the DSS group. Meanwhile, in the normal colonic mucosa (Fig. 3D1), the CDX-2 positive epithelial cells located densely in the colonic glands. For the DSS group (Fig. 3D2), at 7d after termination of DSS intake, there were few CDX-2 positive epithelial cells in
the inflammatory scar of the colonic mucosa. For the DSS + *B. subtilis* fermented milk group (Fig. 3D3), at 7d after termination of DSS intake, a lot of CDX-2 positive epithelial cells were observed in the regenerative glands of the colonic mucosa. Mucin2 is a major intestinal O-glycosylated protein secreted by goblet cells. This secreted protein formed the important physiological barrier for host defence against pathogenic bacteria. For the normal group, large amounts of Mucin2 positive goblet cells were observed in the small intestinal mucosa (Fig. 3E1) and the colonic mucosa (Fig. 3F1). For the DSS group, only few Mucin2 positive goblet cells were observed in the remaining villi in the small intestine(Fig. 3E2) and the scars in the colon(Fig. 3F2). For the DSS + *B. subtilis* fermented milk group. Large amounts of Mucin2 positive goblet cells were observed in the regenerative mucosa in both the small intestine (Fig. 3E3) and the colon (Fig. 3F3). These results suggested that the integrative epithelial and mucous barrier covering the intestinal mucosa were recovered due to intake of the *B. subtilis* fermented milk. In accordance with the above results, the western blotting results (Fig. 3G, H) showed that the relative expression levels of Lgr5, CDX2 and Mucin2 in the DSS group were significantly lower than those of the normal group. The relative expression levels of these proteins in the DSS + *B. subtilis* fermented milk group were significantly higher than those of the DSS group. These results indicated that the *B. subtilis* fermented milk could induce the expression of these proteins, resulting in the regeneration of the epithelium lining the intestinal mucosa injured by DSS induced IBD.

**Effect of the B. subtilis fermented milk on the expression of ZO-1 and Villin**

ZO-1 is the marker protein of tight junction of the intestinal epithelium, and Villin is the marker protein of the microvilli located in the free surface of the epithelial cells. These proteins play an important role in maintaining the integrity of epithelium barrier. The image of IHC staining for ZO-1 in Fig. 4A1 showed that, in the normal small intestinal mucosa, the villi and the crypts arranged compact, and the ZO-1 showed dotted line like distribution (the dots represented tight junction between the epithelial cells) along the surface of the villi and the crypts. For the DSS group (Fig. 4A2), at 7d after termination of DSS intake, ZO-1 distributed dispersively in the residual epithelium of the small intestinal mucosa. However, in the DSS + *B. subtilis* fermented milk group (Fig. 4A3), the ZO-1 distribution was similar to that of the normal small intestine, and showed dotted line like distribution along the surface of the newborn villi, which suggested that the new tight junctions between the regenerative epithelial cells were reconstructed. Meanwhile, in the normal colonic mucosa (Fig. 4B1), the ZO-1 was located mainly at the membrane of the epithelial cells. For the DSS group (Fig. 4B2), there was not obvious ZO-1 positive cell in the colonic mucosa due the formation of inflammatory ulcer and scar in the colonic mucosa. However, for the DSS + *B. subtilis* fermented milk group (Fig. 4B3), the ZO-1 was located mainly at the membrane of the regenerative epithelial cells of the colonic mucosa. For IHC staining of the Villin, which showed strip like distribution on the surface of the normal small intestinal mucosa due to its distribution in the microvilli of the epithelial cells, the strip represented the straited border consisted of the microvilli (Fig. 4C1). In the DSS group, Villin distributed on the surface of the residual villi of the small intestinal mucosa (Fig. 4C2). However, in the DSS + *B. subtilis* fermented milk group, Villin positive staining formed integrative strip enclosing the surface of the regenerative villi of the small intestinal mucosa (Fig. 4C3). Meanwhile, in the
normal colonic mucosa, Villin was distributed at the surface of the epithelium (Fig. 4D1). In the DSS group, there was not Villin positive cells in the scar of the colonic mucosa (Fig. 4D2). In the DSS + B. subtilis fermented milk group, Villin positive microvilli showed strip like distribution on the surface of the colonic epithelium (Fig. 4D3). According to the western blotting results (Fig. 4E, F), the relative expression levels of ZO-1 and Villin in the DSS group were significantly lower than those of the normal group. The relative expression levels of ZO-1 and Villin in the DSS + B. subtilis fermented milk group were significantly higher than those of the DSS group. These results suggested that the oral intake of the B. subtilis fermented milk could induce the small intestinal and colonic epithelium to over-express the ZO-1 and Villin, which promoted the formation of tight junction between the intestinal epithelial cells and maintained the integrity of the epithelium barrier.

Effect of the B. subtilis fermented milk on the intestinal flora of DSS-induced IBD model animals

The etiology of IBD remains unknown. However, one of the main causes is likely related to the imbalance in the gut microbiota. Changes in the microbiota have been observed in previous studies, and decreased bacterial diversity and increased bacterial instability were verified in patients with IBD compared with that in healthy individuals. In this study, DSS definitely disrupted the balance of the intestinal flora and altered the diversity and composition of the gut microbiota. The α-diversity Shannon index and Chao index (Fig. 5) showed that the flora diversity of the B. subtilis fermented milk group was significantly higher than that of the normal group, which suggested that the introduction of B. subtilis enhanced the diversity and the balance of the intestinal flora. The Shannon index and the Chao index of the intestinal microbiota was significantly lower in the DSS group than those of the normal group. It is suggested that DSS-induced IBD is related with enteric dysbacteriosis. The Shannon index and the Chao index of the DSS + B. subtilis fermented milk group were significantly higher than those of the DSS group, which suggested that intake of B. subtilis could maintain the intestinal microbiota diversity.

The abundance heatmap of the flora on genus level was shown in Fig. 6. In B. subtilis fermented milk group, the abundance of Bacillus, Enterococcus, Alloprevotella, Ruminococcus and Buttiauxella were significantly higher than in normal group. In DSS group, the abundance of multiple bacteria (including Alistipes, Rikenella, Barnesiella, Macellibacteroides, and Lactobacillus) were significantly decreased in comparison with the normal group, while the abundance of the Escherichia and Bacteroides increased dramatically. The flora composition of DSS + B. subtilis fermented milk group was very different from that of DSS group. The abundance of Bacillus, Alloprevotella and Ruminococcus were higher than those of normal group and DSS group. The abundance of Alistipes and Lactobacillus in DSS + B. subtilis fermented milk group were much higher than in the DSS group, but slightly lower than in normal group.

The dominant bacterial on family level is shown in Fig. 7, the dominant bacterial in the normal group included Lactobacillae, Porphyromonadaceae, Bacteroidaceae, Lachnospiraceae, and Desulfovibrionaceae. In the DSS group, the dominant families were Lachnospiraceae and Bacteroidaceae. Compared to the DSS group, the fractions of Lachnospiraceae and Bacteroidaceae were
significantly reduced in DSS + B. subtilis fermented milk group, and the fraction of Lactobacillae was largely increased.

The difference analysis in mean proportions (Fig. 8A) showed that the abundance of Bacillus, Barnesiella, Alistipes, and Saccharibacteria was higher in the DSS + milk group than in the DSS group. According to the LEfSe algorithm analysis (Fig. 8B), the abundance of Clostridiales, Turicibacter, and Allobaculum was significantly higher in DSS group than in normal group. For DSS + B. subtilis fermented milk group, the abundance of Clostridiaceae_1, Bacillus, Bacillales, Bacillaceae_1, Phascolarctobacterium, and Selenomonadales were significantly higher than those of the other groups. The abnormal high fractions of Clostridiales, Turicibater, and Allobaculum in the DSS group were regulated in the DSS + B. subtilis fermented milk group. These results suggested that oral intake of B. subtilis fermented milk could efficiently increase the flora diversity of the intestinal microbiota and restore the balance of gut flora which was disturbed by DSS.

**Discussion**

DSS is widely used to construct the IBD animal model. It has been assumed in most former researches that DSS induces epithelial damage mainly in the colon and pathological change in the small intestine is ignored. In this research, however, it was observed that DSS induced damage in both the small intestine and the colon when it was given orally in drinking water. Local mucosa of the small intestine and the colon was damaged by ulcer and hemorrhagic necrosis, along with inflammatory cell infiltration. This result was in agreement with the report from Elsheikh et al. and suggested that the DSS induced IBD was more similar to CD in the pathological features. In comparison with the colon, the small intestine is longer, with larger mucosa surface, which is favourable for nutrition adsorption. The damage of small intestine mucosa would lead to innutrition, which could cause various mobidness including weight loss and the decrease of immunological defence. Thus, the protection of the small intestinal structure and function is more important than protection of the colon. Based on the pathological characteristics of IBD, the prevention and curation of IBD should focus on the inhibition of inflammatory reaction and the protection of the mucosa both in the small intestine and in the colon from inflammatory injury. Because the CD, especially small-bowel Crohn's disease, possessed the higher disease severity and more extensive injury in the intestinal mucosa (including small intestine and colon) than UC, the protection of small intestine from inflammatory damage and promotion of the mucosal healing in the small intestine of IBD are more effective for prevention and curation of this disease especially for small-bowel Crohn's disease. Meanwhile, the promotion of epithelial stem cell proliferation and differentiation, and the reconstruction of the integrate mucosa barrier are essential for recovery from IBD. Besides the traditional therapy of anti-inflammatory drugs, the application of regulators for the gut flora is a novel therapeutic strategy in preventing recurrence and ameliorating refractoriness of IBD.

This study focused on the protective and repairing effects of the B. subtilis fermented milk on both small intestinal and colonic mucosa in the DSS-induced IBD mouse model, and aimed at exploring the action mechanisms of the B. subtilis fermented milk. The results indicated that oral intake of DSS could induce
extensive injury of the small intestinal mucosa and the colonic mucosa. In the active phase of the DSS-induced IBD, the major pathological characteristics included necrosis of the epithelium and the ulcers in the mucosa. The damage in the colon was more severe than in the small intestine. In the recovery stage, the injury in the small intestine could partially recover automatically, while the ulcers of the colonic mucosa were replaced by inflammatory scars. For the small intestine, in the active phase (DSS inducing phase), oral intake of the \textit{B. subtilis} fermented milk could prevent the inflammatory injury; And in the recovery phase (after DSS inducing), the \textit{B. subtilis} fermented milk could promote the repairing of the injury and then completely reconstruct the microstructure of the mucosa. For the colon, in the active phase, oral intake of the \textit{B. subtilis} fermented milk could lessen the ulcer of the mucosa; And in the recovery phase, oral intake of the \textit{B. subtilis} fermented milk could inhibit the formation of the scars in the colonic mucosa, and promote the regeneration of the epithelium. These results suggested that \textit{B. subtilis} fermented milk possessed the double-function of both prevention and curation for DSS-induced IBD. Meanwhile, the results also showed that oral intake of the \textit{B. subtilis} fermented milk could induce the goblet cells to secret more mucus which was stained blue via alcian blue. The mucus secreted by the goblet cells was important for construction of an integrate mucus barrier, which can protect the deep area of the mucosa from the infiltration of the pathogenic bacteria, and thus inhibit the local inflammatory reaction.\textsuperscript{42,43}

In this research, the action mechanisms of the \textit{B. subtilis} fermented milk for treatment of DSS-induced IBD were explored. The \textit{B. subtilis} fermented milk could inhibit the MPO$^+$ neutrophil infiltration and the expression of pro-inflammatory cytokine TNF, and promote the expression of the anti-inflammatory cytokine IL-10 in the intestinal mucosa, which reduced local inflammatory injury of the intestinal mucosa. It has been reported that TNF was over-expressed in the intestinal mucosa and could lead apoptosis of the intestinal epithelial cells (IECs) in IBD.\textsuperscript{44–46} However, anti-inflammatory cytokine IL-10 exerted essential functions to maintain tissue homeostasis during infection and inflammation through restriction of excessive inflammatory responses and promotion of tissue repairing mechanisms.\textsuperscript{47,48} IL-10 could secreted by both immune cells and IECs.\textsuperscript{49,50} IL-10 binds to a specific receptor on IECs and may regulate the contribution of epithelial cells to the inflammatory and immune response in the digestive tract via auto-secretion pathway.\textsuperscript{51–54} Many experimental results indicated that IL-10-deficient mouse has susceptibility to IBD, and over-expression of IL-10 was related to good therapeutic effect of drugs for IBD.\textsuperscript{55–57} In this study, the results of immunohistochemical staining and western-blotting indicated that oral intake of the \textit{B. subtilis} fermented milk could inhibit expression of TNF and promote over-expression of IL-10 in the IECs, which might be an important action mechanism of the \textit{B. subtilis} in treatment of DSS-induced IBD.

The results of IHC and western blotting for the marker of intestinal stem cells (ISCs) Lgr5 and epithelial marker CDX2 indicated that oral intake of the \textit{B. subtilis} fermented milk could promote epithelial regeneration via protection of the intestinal stem cells (ISCs) from inflammatory injury and induced proliferation of the ISCs. ISCs can proliferate and differentiate into the intestinal epithelial cells and goblet cells.\textsuperscript{58–62} DSS leaded to the loss of Lgr5$^+$ cells, however, \textit{B. subtilis} could increase Lgr5$^+$ ISCs
and then result regeneration of the epithelium lined the intestinal mucosa injured by DSS induced IBD. In addition, the *B. subtilis* fermented milk could induce the small intestinal and colonic epithelium to over-expression the CDX2. CDX2 is an intestinal specific transcription factor located in the nuclei of IECs and modulates a diverse set of cellular behaviours, including cell proliferation and differentiation, and cell adhesion and migration. CDX2 is an essential regulator of intestinal epithelium homeostasis.\(^6\) TNF-α could impair the functions of CDX2 in IBD leading to the mucosal injury.\(^6\) Inducing expression of CDX2 by the *B. subtilis* fermented milk suggested that the *B. subtilis* could maintain intestinal epithelium homeostasis via its inhibition against the TNF-α or via direct action to stimulate expression of CDX2. Meanwhile, oral intake of *B. subtilis* fermented milk could promote expression of Mucin2 in the goblet cells and expression of Villin in the epithelial cells. Mucins are the main components of mucus, which is secreted by goblet cells and forms a protective homeostatic barrier between the resident microbiota and the underlying immune cells in the colon.\(^6\) It has been reported that 5 weeks after birth, MUC2 knockout animals develop spontaneous colitis and display increased susceptibility to experimental DSS colitis.\(^6\) Plaisancié et al. reported that a novel bioactive peptide produced from bovine β-casein in yoghurts could induce expression of the gel-forming MUC2 mucin in the human intestinal mucus-producing cells (HT29-MTX) and enhanced the number of goblet cells and Paneth cells along the small intestine.\(^6\) In this study, over-expression of Mucin2 by oral intake of *B. subtilis* fermented milk suggested that *B. subtilis* or/and some bioactive peptide produced from bovine β-casein in the fermented milk might play a role in treatment of DSS-induced IBD via the protective function of Mucin2 as well. Villin is an actin regulatory protein expressed in the intestinal epithelium and possesses the epithelial cell-specific anti-apoptotic function.\(^6\) Absence of Villin predisposes mice to DSS-induced colitis by inducing apoptosis of the IECs.\(^6\) Inducing expression of Villin suggested that *B. subtilis* fermented milk could play the anti-apoptotic role in treatment of DSS-induced IBD. More importantly, oral intake of the *B. subtilis* fermented milk could promote expression of ZO-1 which showed dotted line like localization on the membrane of the IECs. The proteins of tight junction, ZO-1 and ZO-2 can bind directly to F-actin and other cytoskeletal proteins, and these proteins are relevant both to cellular organization and epithelial morphogenesis.\(^7\) It has been reported that the expression of ZO-1 was significantly higher in the patients with quiescent UC with mucosa healing compared with those without mucosal healing, and the loss of ZO-1 could increase permeability of the intestinal epithelium and promoted the development of significant intestinal inflammation in animals with DSS colitis.\(^7\) Peng et al. reported that the probiotic *B. subtilis CW14* could reduce disruption of the epithelial barrier and toxicity of ochratoxin A to Caco-2 cells via improving ZO-1 protein expression.\(^7\) In this study, the high expression level and the dotted-line like distribution of ZO-1 along the surface of the IECs in the normal group and DSS + *B. subtilis* fermented milk group suggested that ZO-1 participated in the construction of the tight junction between the intestinal epithelial cells. These results suggested that *B. subtilis* could promote reconstruction of the epithelium barrier which prevented pathogenic bacterial invasion and protected the intestinal mucosa from inflammatory injury in DSS-induced IBD.
The inflammatory injury in the intestine was reported to be related with the imbalance of the intestinal flora and reduction of the abundance and diversity of the gut microbiota. As reported by Sjöberg et al., pyrosequencing revealed that the gut microbiota of patients with ulcerative colitis contained fewer Operational Taxonomic Units (OTU) per individual than the controls, and this reduction in richness of the gut microbiota was observed in *Firmicutes, Actinobacteria, Collinsella, Lactobacillus,* and *Bacillus.* In this research, oral intake of the *B. subtilis* fermented milk increased the species diversity of the normal intestinal microbiota. And the decrease in abundance of *Alistipes, Rikenella, Barnesiella, Macellibacteroides,* and *Lactobacillus* was observed in the DSS-induced IBD models, while the abundance of *Escherichia* and *Bacteroides* increased. According to the LEfSe analysis results, *Clostridiales* and another genus in *Clostridium (Clostridium_IV)* were the most significantly increased genus in the DSS group and the DSS + milk group. Some species in the *Clostridiurn* genus were reported to be opportunistic pathogens associated with intestinal infection. *Clostridiurn difficile* was reported to be related with diarrhea. In accordance with our results, it was reported in several researches that the relative abundance of *Bacteroides* was higher in DSS-induced IBD models. On the other hand, the results suggested that the *B. subtilis* fermented milk could increase the total species and diversity of the gut microbiota which were reduced by DSS-induced IBD. Meanwhile, the fractions of *Lactobacillae* and *Porphyromonadaceae* in the DSS + *B. subtilis* fermented milk group were increased to the similar level of the normal group. Oral intake of the *B. subtilis* fermented milk significantly increased the abundance of *Bacillus, Barnesiella, Alistipes,* and *Saccharibacteria,* and the total OTU detected was largely increased. Another genus in *Clostridiaceae* (different from the genus detected in other groups) was detected in DSS-*B. subtilis* fermented milk group, but the LDA score was lower than the *Clostridiales* genus detected in the DSS group. Meanwhile, the abundance of *Escherichia,* which was reported to be related with inflammatory reactions, was reduced. The dramatic increase of *Bacillus* abundance after oral intake of the *B. subtilis* fermented milk suggested that *B. subtilis* was successfully implanted in the intestine. Among the species increased through *B. subtilis* intake, *Barnesiella* and *Alistipes* have been approved beneficial to intestinal health. *Alistipes* was reported to reduce the inflammatory reaction in the intestine and regulate the lipid metabolism. And *Barnesiella* could facilitate cyclophosphamide, which possesses anti-cancer activity. Besides, the abundance of short-chain fatty acid (SCFA)-producing *Ruminococcus* was significantly higher in the DSS + *B. subtilis* fermented milk group than in the other groups, and it was reported that the absence of *Ruminococcus* was related with CD. According to the results in this study, oral intake of *B. subtilis* fermented milk could efficiently increase the gut flora diversity of the intestinal microbiota and restore the balance of gut flora which was disturbed by DSS; The *B. subtilis* in the fermented milk might play the important role in the prevention and curation of IBD, via regulation of the intestinal flora.

In conclusion, this research focused on the prevention and curation effects of the *B. subtilis* fermented milk on the DSS-induced IBD and exploring the action mechanisms, including inhibition of inflammation, promotion of reconstruction of intestinal mucosal barrier, and regulation of the intestinal flora. It was demonstrated that oral intake of the *B. subtilis* fermented milk could reduce the inflammatory injury of both the small intestinal and colonic mucosa, and promote the epithelial regeneration and reconstruction...
of the intestinal mucosa barrier. Oral supplement of the *B. subtilis* could increase the total species and diversity of the bowel microbiota and regulate the gut flora balance which was disturbed by DSS-induced IBD. The results indicated that oral intake of the *B. subtilis* fermented milk could prevent and cure the DSS-induced IBD in mice. The *B. subtilis* fermented milk would be a potential novel functional food for the application in the therapy of IBD. However, it remains to be further explored what bioactive ingredients (the probiotic *B. subtilis*, its metabolites or these cooperative factors) in the *B. subtilis* fermented milk possessed the functions of prevention and curation of IBD. In addition, the molecular signaling pathway related to the function of *B. subtilis* in treatment of IBD is worthy of further study.

Materials And Methods

Materials

DSS (molecular weight 36,000–50,000) was purchased from MP Biomedicals, LLC, (CA, USA); Antibodies, including Anti-MPO, Anti-TNF-α, Anti-IL-10, Anti-Lgr5, Anti-CDX2, Anti-Mucin2, Anti-ZO-1, Anti-Villin, Anti-β-Actin and Anti-goat IgG-HRP, Anti-rabbit IgG-HRP and Anti-mouse IgG-HRP, RIPA lysis buffer and BCA Protein Assay Kit were purchased from Boster Biological Technology Co., Ltd (Wuhan, Hubei, China); Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (CA, USA); Pierce ECL Plus western blotting substrate was purchased from Thermo Fisher Scientific (MA, USA); UHT whole milk was purchased from Mengniu Dairy (Hohhot, Inner Mongolia, China); The *B. subtilis* strain JN0126 was isolated from natto in our experiment.

Preparation of the *B. subtilis* fermented milk

The *B. subtilis* strain JN0126 was used to prepare the *B. subtilis* fermented milk. The fermentation substrate milk was composed of the UHT whole milk and 2% sucrose (w/w). The substrate milk was heated at 95°C for 5 min. As cooled down to 40°C, the *B. subtilis* strain JN0126 amplified in the normal medium was inoculated to the substrate milk at a concentration of 10⁶ CFU/ml. The milk was fermented at 41°C for 8 hours and a firm curd was formed. The final CFU of *B. subtilis* in the fermented milk was 6 × 10⁸ CFU/ml. The *B. subtilis* fermented milk was stored at 4°C for no more than 24 h before usage.

Establishment of the DSS-induced IBD model and the experimental design

In this study, all animal experimental protocols were approved by the Ethics Committee of Jiangsu University for the use of experimental animals and conformed to the Guide for the Care and Use of Laboratory Animals. The male C57BL6/J mice, 8-week-old and weighing 22–23 g, were raised at room temperature (25 °C ± 2 °C) and the mice were exposed to a 12 h light/dark cycle with free access to standard rodent chow and water in the barrier facility of Animal Center of Jiangsu University. Then, the 100 mice were divided into 5 groups (20/group, Table 1 showed the experimental procedures): (1) The control (normal) group, was fed with standard chow and drank sterile water; (2) The *B. subtilis* fermented milk group, was fed with standard chow and drank sterile water, in addition, 300 ul the *B. subtilis*...
fermented milk was orally given by gavage twice daily for 21 days; (3) The DSS group, was fed with standard chow for 21 days and drank sterile water during the first 7 days and the third 7 days. For inducing IBD, during the second 7 days, the normal drinking water was replaced by 3.5% DSS (in drinking sterile water, ad libitum access). In addition, 300 ul normal saline was orally given by gavage twice daily for 21 days; (4) The DSS + milk group, was fed with standard chow for 21 days and drank sterile water during the first 7 days and the third 7 days. For inducing IBD, during the second 7 days, the normal drinking water was replaced by 3.5% DSS (in drinking sterile water, ad libitum access). In addition, 300 ul substrate milk was orally given by gavage twice daily for 21 days; (5) The DSS + \textit{B. subtilis} fermented milk (FM) group, was fed with standard chow for 21 days and drank sterile water during the first 7 days and the third 7 days. For inducing IBD, during the second 7 days, the normal drinking water was replaced by 3.5% DSS (in drinking sterile water, ad libitum access). In addition, 300 ul \textit{B. subtilis} fermented milk was orally given by gavage twice daily for 21 days for prevention and cure the IBD. During the experimental time, the health and symptom of the animals including diarrhea and blood in fecal matter was monitored twice a day. The body weight was recorded every day. 5 mice from each group were executed at 14 d (the active phase of IBD, after orally intake of DSS for 7 days ), and the other 15 mice were executed at 21 d (the recovery phase of IBD, after the termination of DSS intake for 7 days) of the experiment. The intestines were taken out from the abdominal cavity, their gross appearance were observed, and then washed with phosphate-buffered saline (PBS). These intestine samples were prepared for histological observation, western blotting assay, and the intestinal contents were used for the analysis of intestinal flora in the following experiments.

**Table 1. The group design of the animal experiment**

| Groups                                      | 0-7d                        | 8-14d                      | 15-21d                      |
|---------------------------------------------|-----------------------------|----------------------------|-----------------------------|
| Normal                                      | Normal drink water          |                            | Standard chow               |
| \textit{B. subtilis} fermented milk         |                            | Normal drink water         |                            |
| DSS                                         | Normal drink water          | 3.5% DSS in drink water    | Normal drink water          |
| DSS+milk                                    | Standard chow + 300 ul \textit{B. subtilis} fermented milk twice daily by oral gavage |
| DSS+\textit{B. subtilis} fermented milk     | Standard chow + 300 ul fermentation substrate milk twice daily by oral gavage |

**Assay for disease severity evaluation**

**Disease activity index assess**
The disease activity index (DAI) was adopted to evaluate the severity of colitis. The body weight and shape and consistency of stools of the animals were checked every day. The DAI scoring system was defined as follows. For weight, 0 indicated no weight loss, 1 indicated 5–10% weight loss, 2 indicated 10–15% weight loss, 3 indicated 15–20% weight loss, and 4 indicated greater than 20% weight loss. For stool, 0 indicated normal stool, 2 indicated loose stool, and 4 indicated diarrhea. For bleeding, 0 indicated no blood, 2 indicated the presence of blood, and 4 indicated gross blood. For observation of the disease progression, the DAI scoring was performed daily from the 8 day to 21 day in the experiment.

**Appearance observation of the intestines**

The mice were deeply anaesthetized with pentobarbital sodium (200 mg/kg, intraperitoneal injection) and then the small intestine and colon were taken out from the abdominal cavity. These samples were photographed and the their appearance, including the shape integrity, surface gloss and elasticity, was observed to assess preliminarily the severity of injury and bleeding in the intestines caused by DSS-induced IBD. The length of the colons of the animals in different groups were recorded to compare the disease severity of DSS-induced IBD among these groups.

**Histology observation of the ileum and colon**

The samples of ileum and colon taken out from the DSS-induced IBD model animals in the active phase and the recovery phase of IBD were fixed with paraformaldehyde. After dehydrated with gradient ethanol, the samples were embedded in paraffin and then sectioned (5 µm thick). The sections were stained with eosin and haematoxylin (H-E staining). In addition, the sections were stained with alcian blue for observation of the goblet cells (which contained mucus) in the mucosa of the intestines.

**Immunohistochemical staining**

After termination of DSS administration for 7 days, all mice were sacrificed, and intestines including the ileum and colon were taken out and then fixed in 4% paraformaldehyde. After dehydrated with gradient ethanol, the samples were embedded in paraffin and then sectioned (5 µm thick) for immunohistochemical staining (IHC). For observation of the infiltration of neutrophils, the inflammatory reaction, and the cellular distribution of pro-inflammatory cytokine and anti-inflammatory cytokine in the intestinal mucosa, the antibodies against the neutrophil marker MPO, pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 were used to stain the sections of ileum and colon of IBD model animals. Meanwhile, to observe the distribution of intestinal stem cells in the mucosa, the epithelial regeneration and reconstruction of the mucosal barrier after the treatment, the specific proteins, including the intestinal stem cells marker Lgr5, the goblet cells marker Mucin2, the intestinal epithelial function proteins CDX2 and Villin and tight junction protein ZO-1, were stained by IHC using the specific antibodies. In the procedures of IHC, the second antibodies were the corresponding IgG-HRP, and the positive staining was showed brown via DAB. The negative control test of IHC was carried out via replacement of specific antibodies against these proteins by the normal serum of rabbit, goat or mouse.

**Western blotting**
At the end of experiments of the DSS-induced IBD model and its treatment, the samples of the same long (5 cm) distal ileum and colon in the normal group, DSS + milk group and DSS + *B. subtilis* fermented milk group were collected and washed twice with PBS. For extraction of the tissue proteins, the samples were lysed with RIPA lysis buffer (100 mg/ml) containing the protease inhibitor cocktail on ice for 30 min and ultrasonicated at 4 °C, and then centrifuged at 5000 g for 5 min. Protein concentrations of the supernatant were quantified with BCA assays kit and then mixed with equal amounts of loading buffer and denatured by heating at 100 °C for 5 min. The proteins from these samples (as equal amount) were loaded and separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to PVDF membranes. The membranes were blocked with blocking solution for 1 h, and then incubated with primary antibodies against target proteins, including MPO, TNF-α, IL-10, Lgr5, CDX2, Mucin2, ZO-1, Villin, and β-Actin (as standard control) for 12 h at 4 °C. For showing of the bands of these target proteins, the membranes were incubated with secondary antibodies conjugated horseradish peroxidase (HRP), and visualized using a Pierce ECL Plus substrate, and then scanned with Typhoon 9400 Variable Mode Imager (Amersham Biosciences). The ratio of gray scale between target protein band and β-actin band was calculated as the relative expression level of the target protein. Each experiment was repeated at least three times for statistical analysis (n = 5).

**Intestinal flora analysis**

The mice were deeply anaesthetized with pentobarbital sodium (200 mg/kg, intraperitoneal injection) and the abdominal cavity was open. The total colons of 5 mice of each group (5 groups) were taken out. The total content of the colon was washed out with PBS and stored at -80 °C. Total genomic DNA was extracted from the colonic content using a E.Z.N.A.Soil DNA Kit (Omega, USA). The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified for species classification by PCR with following primers: the forward primer (CCTACGGGNGGCWGCAG) and the reverse primer (GACTACHVGGGTATCTAATCC). The AMPure XP beads to remove the free primers and primer dimer species in the amplicon product. Samples were delivered to Sangon BioTech (Shanghai, China) for library construction using universal Illumina adaptor and index. Sequencing was performed using the Illumina MiSeq system (Illumina MiSeq, USA). The sequences were clustered into operational taxonomic units of at least 97% identity. The following classification of the taxonomic and analysis were operated based on the mothur software package according to the standard pipeline described on the mothur website.

**Statistical analysis**

All data were presented as the mean ± standard deviation (SD). Statistical analysis was performed by SPSS 13.0 statistical software. The two-way analysis of variance (ANOVA) was used to analyze the difference between groups, and *p* < 0.05 was considered to be statistically significant.

**Declarations**

*Ethics approval*
All animal experimental protocols were approved by the Ethics Committee of Jiangsu University for the use of experimental animals and conformed to the Guide for the Care and Use of Laboratory Animals.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

No individual person’s data is included for publication.

**Data availability statement**

All the experimental data underlying this article are available on request.

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**Author contributions**

X.Z.: Data collection, data analysis and initial manuscript drafting

Y.T.: Study design, data analysis and initial manuscript drafting

X.L.: Data analysis, manuscript reviewing and revision

J.W.: Data collection and data analysis

Y.W.: Data collection and data analysis

R.Y.: Study design, data analysis, manuscript reviewing and revision

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