

# *Saccharomyces boulardii* modulates necrotizing enterocolitis in neonatal mice by regulating the sirtuin 1/NF- $\kappa$ B pathway and the intestinal microbiota

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**Abstract.** Exaggerated inflammatory response and gut microbial dysbiosis play a crucial role in necrotizing enterocolitis (NEC). The probiotic *Saccharomyces boulardii* (SB) is a yeast that has a beneficial effect on NEC; however, the association between its protective effects and the regulation of the inflammation-related sirtuin 1 (SIRT1)/nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway and gut microbiota in NEC is unknown. In the present study, the NEC model was established by artificial feeding and lipopolysaccharide (LPS), hypoxia and hypothermia stimulation. Mice were divided into normal, control (artificial feeding), NEC and NEC + SB groups. Hematoxylin and eosin staining demonstrated that SB improved the pathological damage of the intestine caused by NEC in neonatal mice. Furthermore, downregulation of SIRT1 and upregulation of NF- $\kappa$ B expression were confirmed by immunofluorescence staining, western blotting and reverse

transcription-quantitative PCR (RT-qPCR) in NEC mice. SB treatment concurrently inhibited the NEC roles on the SIRT1 and NF- $\kappa$ B pathway at both the protein and mRNA levels. Deletion of SIRT1 [SIRT1 knockout (KO)] in the intestine abolished all the effects of SB in NEC mice, including protection of pathological damage and inhibition of the SIRT1/NF- $\kappa$ B pathway activation. The abundance of gut microbial composition, as determined by RT-qPCR, was significantly decreased in the control group compared with the normal group. A further decrease in microbiota abundance was observed in the NEC group, and SB administration significantly improved the enrichment of gut microbiota in neonatal mice with NEC. As anticipated, the increased abundance of gut microbiota modulated by SB was markedly reduced in SIRT1KO NEC mice. The present study revealed that the protective role of SB on NEC was associated with the SIRT1/NF- $\kappa$ B pathway and gut microbiota regulation.

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**Abbreviations:** Bact, *Bacteroides*; Clept, *Clostridium leptum*; DAPI, 4,6-diamidino-2-phenylindole; Erec, *Eubacterium rectale/Clostridium coccoides*; H&E, hematoxylin & eosin; IL-6, interleukin-6; Lact, *Lactobacillus*; LPS, lipopolysaccharide; MIB, mouse intestinal *bacteroides*; NEC, necrotizing enterocolitis; NF- $\kappa$ B, nuclear factor- $\kappa$ B; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SB, *Saccharomyces boulardii*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; SFB, segmented filamentous bacteria; SIRT1, sirtuin 1; sp., species; TNF- $\alpha$ , tumor necrosis factor- $\alpha$

**Key words:** *Saccharomyces boulardii*, necrotizing enterocolitis, sirtuin 1, nuclear factor- $\kappa$ B, gut microbiota

## Introduction

Necrotizing enterocolitis (NEC) is considered as one of the most devastating diseases in neonates, especially those with markedly low birth weight (<1,500 g) (1). NEC is characterized by necrosis of intestinal mucosa and submucosal tissue, and is associated with various pathogenic mechanisms, such as premature birth, hypoxia, hyperosmotic artificial (namely formula) feeding, imbalance of the gut microbial ecosystem, infection and inflammatory response (1,2). NEC can be classified into three stages: i) Stage 1 (suspect) is defined by temperature instability, lethargy, apnea, bradycardia and mild abdominal distension; ii) Stage 2 (definite) may be recognized as having the aforementioned signs and symptoms plus persistent occult or gross gastrointestinal bleeding and/or marked abdominal distension; iii) Stage 3 (advanced) is diagnosed as having the aforementioned signs and symptoms plus deterioration of vital signs, evidence of septic shock and/or marked gastrointestinal hemorrhage (3). The incidence of NEC in the USA neonatal intensive care units is 3-11% (4-6). From 1999 to 2009, the incidence of NEC in >600 hospitals in the USA was significantly increased, while all the other neonatal

morbidities exhibited a stable or declining trend (4,6,7). There is no effective prevention or treatment of NEC in the clinic at present. Oral probiotic preparations, such as *Bifidobacterium* and *Lactobacillus* species, have been used to improve the gut microbial composition in neonates with NEC (8).

The non-pathogenic yeast *Saccharomyces boulardii* (SB) has been reported to be effective in the prophylaxis and treatment of a wide range of enteropathies (9). It was reported that this probiotic yeast has beneficial effects on enteropathies, such as improving the gut immune response and the intestinal barrier (10,11). In addition, previous studies have suggested that SB improves intestinal necrosis in neonatal mice with NEC; however, the majority of the mechanisms require further elucidation (12-14).

Sirtuin 1 (SIRT1) is a member of the sirtuin family, and it is widely expressed in a variety of organisms, from yeast to mammals (15). Previous results indicated that SIRT1 is closely associated with inflammation (16). Several inflammatory transcription factors such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) are modulated by SIRT1. Overexpression of SIRT1 has been reported to enhance NF- $\kappa$ B p65 subunit deacetylation, and suppress NF- $\kappa$ B transcription and activity (17). Once NF- $\kappa$ B is activated, it upregulates the expression of downstream cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6), which will eventually lead to an inflammatory response (18). However, the role of the SIRT1/NF- $\kappa$ B pathway in neonatal mice with NEC, and whether it can be regulated by SB treatment, is still not fully understood.

Furthermore, the main microbial composition in the intestine of mice is *Eubacterium rectale/Clostridium coccoides*, *Clostridium leptum*, *Lactobacillus species (sp.)*, *Bacteroides sp.*, mouse intestinal *Bacteroides* and segmented filamentous bacteria, which are important for the intestinal microenvironment (19). Therefore, whether modulation of the gut microbiota is responsible for the role of SB on neonatal mice with NEC should be investigated.

The present study revealed that neonatal mouse intestinal epithelial SIRT1 and its downstream NF- $\kappa$ B were critically involved in the protective role of SB in mice with NEC. Furthermore, the modulation of the gut microbiota was also associated with the SB effect on NEC.

## Materials and methods

**Animals.** A total of 76 newborn male and female C57BL/6 mice (8 weeks old, weight 2-4 g) were obtained from the Experimental Animal Center of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Intestinal epithelial specific SIRT1 knockout (KO) newborn mice (SIRT1<sup>KO</sup>, villin-cre<sup>+</sup>, SIRT1<sup>flox/flox</sup>) and their littermate Flox controls (villin-cre<sup>+</sup>, SIRT1<sup>flox/flox</sup>) on a C57BL/6 background were generated as previously described (20). The mice were socially housed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Edition, 2011). All animal experiments were approved by the Ethics Committee on Animal Care of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

**Artificial feeding.** The neonatal mice were placed into an incubator with a constant temperature of 36°C and a humidity of 55% on the day of birth, and were artificially fed using a formula

previously described (Table I) (21). The neonatal mice were intubated with a sterile 1.9F silicone tube (BD Biosciences) every 4 h. The skin around the mouth of the neonatal mice was cleaned with 0.9% sodium chloride and 75% ethanol solution before feeding. The first feeding volume was 0.1 ml, and 0.1-0.3 ml was added every 24 h.

**Induction of NEC model and *Saccharomyces boulardii* (SB) treatment.** Mice with NEC were established as previously described (22). Briefly, neonatal mice were fed with lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA) at 2 mg/kg in addition to artificial feeding for 3 days. Furthermore, neonatal mice were exposed to hypoxia (99% N<sub>2</sub>) for 1 min in a hypoxic chamber, followed by hypothermia (4°C) for 10 min twice daily for an additional 3 days (23). The NEC model was successfully established when the animals appeared to be severely ill with signs of cyanosis, lethargy and/or abdominal distension, which was similar to the human NEC in stage II and III. The mice continued to be artificially fed for 3 days and then sacrificed by cervical dislocation. During this period, if the mouse exhibited more serious symptoms, such as severe abdominal distention, disappearance of bowel sounds, serious decrease of heart rate, hypothermia, and/or shock, it was sacrificed by cervical dislocation. After establishing the NEC model, the newborn mice were orally administered with SB sachets (>1.3x10<sup>9</sup> CFU/g; Laboratoires Biocodex) once a day at 800 mg/kg/day for 3 days. After the gavage, the neonatal mice were returned to the incubator, and the mice with NEC were sacrificed after 3 day SB treatment. Notably, our previous study indicated that 800 mg/kg SB significantly increased the body weight and survival cycle, and decreased the pathological score of neonatal mice with NEC compared with other SB doses (unpublished data). Therefore, 800 mg/kg SB was used in the present study.

**Material acquisition.** Mice from the normal and control groups were sacrificed 6 days after breastfeeding or artificial feeding. Mice with NEC were sacrificed 6 days after the induction of NEC, while NEC + SB mice were sacrificed 3 days after SB treatment. These samples were all acquired at the same time among the experimental groups. After euthanasia, mice were incised from the median region, and the tissues and blood vessels were separated. Next, the ileocecum (~5 cm) was obtained, fixed in 4% paraformaldehyde for 24 h at room temperature, and embedded in paraffin. In addition, a section of ileocecal intestinal tissue was stored for subsequent use in immunofluorescence staining, western blotting and reverse transcription-quantitative PCR (RT-qPCR).

**Hematoxylin and eosin (H&E) staining and pathological score assessment.** The embedded ileocecal intestinal tissue was sliced at a thickness of 4  $\mu$ m, and stained with an H&E Staining kit (Beyotime Institute of Biotechnology) following the manufacturer's instructions. The diagnosis of NEC was performed based on the assessment of two independent pathologists, who were blinded in this study. NEC was scored based on the severity of the damage from the mucosa to the lamina propria as follows (24): i) 0, intact villi and epithelium with normal tissue structure; ii) 1, mild separation or edema in the submucosa and/or lamina propria; iii) 2, moderate separation or edema in the submucosa and/or lamina propria; iv) 3, severe

Table I. Comparison of AF formula and main components of mouse milk.

Ingredients	Substitute milk (1 l)	Mouse milk (1 l)
Sugar (g)	28.04	26
Protein (g)	100	69-118
Fat (g)	105.7	93-175
Calories (kJ)	5,817.23	5,200-9,300

AF, artificial feeding.

separation or edema in the submucosa and/or lamina propria, and villi shedding; and v) 4, complete absence of epithelial structures and transmural necrosis. A pathological score  $\geq 2$  was classified as NEC.

**Immunofluorescence staining.** The ileocecal tissue sections were fixed for 15 min with 4% paraformaldehyde at room temperature, blocked with 5% BSA (Beyotime Institute of Biotechnology) at room temperature for 30 min, and incubated with primary antibodies (SIRT1; 1:1,000; cat. no. ab220807; Abcam) at 4°C overnight. Following which, tissues were incubated at room temperature for 1 h with donkey anti-mouse IgG Alexa Fluor 350-conjugated secondary antibody (cat. no. A10035) and donkey anti-mouse IgG Alexa Fluor 594-conjugated secondary antibody (cat. no. A32744; 1:1,000; both purchased from Thermo Fisher Scientific, Inc.). Then, the sections were stained with DAPI (Sigma-Aldrich; Merck KGaA) at room temperature for 10 min. After final washing, the coverslips were mounted on slides using 50% glycerin. The sections were then observed using a fluorescence microscope (Olympus Corporation).

**Western blotting.** Cytoplasmic and nuclear proteins were extracted from ileocecal tissues using a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Protein concentrations were measured via bicinchoninic acid assay (Beyotime Institute of Biotechnology). Proteins (40 µg) were loaded onto each lane and separated by 8-12% SDS-PAGE and electro-transferred to nitrocellulose membranes (EMD Millipore). Then, the membranes were probed with antibodies against SIRT1 (cat. no. ab220807), NF-κB (cat. no. ab16502), β-actin (cat. no. ab8226) or lamin B1 (cat. no. ab16048; 1:1,000; all purchased from Abcam) at 4°C overnight, followed by incubation with a LICOR IRDye® 800CW goat anti-mouse IgG secondary antibody (cat. no. 926-32210) and IRDye 680RD goat anti-rabbit IgG (cat. no. 926-32221; 1:10,000; both purchased from LI-COR Biosciences) at room temperature for 2 h. Finally, the signals were detected by Odyssey Infrared Imaging System (LI-COR Biosciences). Digitized images were determined by ImageJ software (v1.47; National Institutes of Health). Protein expression levels were calculated from the ratio of corresponding protein to β-actin (cytoplasmic loading control) or lamin B1 (nuclear loading control) multiplied by 100%.

**RT-qPCR and qPCR.** Total RNA from ileocecal tissues was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The RNA was then subjected to RT to synthesize cDNA using the PrimeScript RT Reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.) at 42°C for 15 min and 85°C for 5 min. Subsequently, 20-µl reactions with SIRT1, NF-κB or β-actin primers (Genewiz, Inc.) were detected using a PikoReal 96 Real-Time PCR system (Thermo Fisher Scientific, Inc.) with SYBR Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative quantitative analysis in the mRNA expression was obtained using the  $2^{-\Delta\Delta C_q}$  method (25) and normalized to β-actin.

Total DNA from the feces and intestinal contents was extracted using the QIAamp DNA Stool Mini kit (Qiagen GmbH). The abundance of gut microbial composition was assessed by qPCR using specific 16S ribosomal DNA (rDNA) primers (Genewiz, Inc.). The data were quantified by calculating the abundance of bacterial group-specific 16S rRNA genes and normalized to total bacterial rRNA genes.

The sequences of the primers were as follows: SIRT1 forward, 5'-CAGCTCTGCTACAATTCATCGCGTC-3' and reverse, 5'-AATCTCTGTAGAGTCCAGCGCGTGTG-3'; NF-κB forward, 5'-ACGAGCAGATGGTCAAGGAG-3' and reverse, 5'-CTTCCATGGTCAGTGCCTTT-3'; TNF-α forward, 5'-TACACCTCACCCACACAGTC-3' and reverse, 5'-CGCACC TCACAGACTGTTTT-3'; IL-6 forward, 5'-GCCAGTTGCCTT CTTGGG-3' and reverse, 5'-CGACTTGTGAAGTGGTATA-3'; β-actin forward, 5'-TAAAGACCTCTATGCCAACACAGT-3' and reverse, 5'-CACGATGGAGGGCCGACTCATC-3'; *Eubacterium rectale/Clostridium coccoides* (Erec) forward, 5'-ACTCCTACGGGAGGCAGC-3' and reverse, 5'-GCTTCT TAGTCAGGTACCGTCAT-3'; *Clostridium leptum* (Clept) forward, 5'-GTTGACAAAACGGAGGAAGG-3' and reverse, 5'-GACGGGCGGTGTGTACAA-3'; *Lactobacillus sp.* (Lact) forward, 5'-GACGGGCGGTGTGTACAA-3' and reverse, 5'-CACCGCTACACATGGAG-3'; *Bacteroides sp.* (Bact) forward, 5'-CACCGCTACACATGGAG-3' and reverse, 5'-GCT GCCTCCCGTAGGAGT-3'; mouse intestinal *Bacteroides* (MIB) forward, 5'-CCAGCAGCCGCGGTAATA-3' and reverse, 5'-CCAGCAGCCGCGGTAATA-3'; segmented filamentous bacteria (SFB) forward, 5'-GACGCTGAGGCATGA GAGCAT-3' and reverse, 5'-GACGCTGAGGCATGAGAG CAT-3'; and total bacteria forward, 5'-ACTCCTACGGGAGGC AGCAGT-3' and reverse, 5'-ATTACCGCGGCTGCTGGC-3'.

**Statistical analysis.** Data were expressed as the means  $\pm$  standard error of mean. Statistical analysis was performed by SPSS 23.0 (IBM Corp.). Significant differences between two groups were analyzed via Student's t-test, multiple comparisons of >two groups were performed using Tukey's honest significant difference test, and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**SB treatment improves NEC-induced intestinal damage.** Based on macroscopic observation of the intestinal specimens, the intestine of normal neonatal mice was bright in color, straight and smooth, without gas accumulation. The color

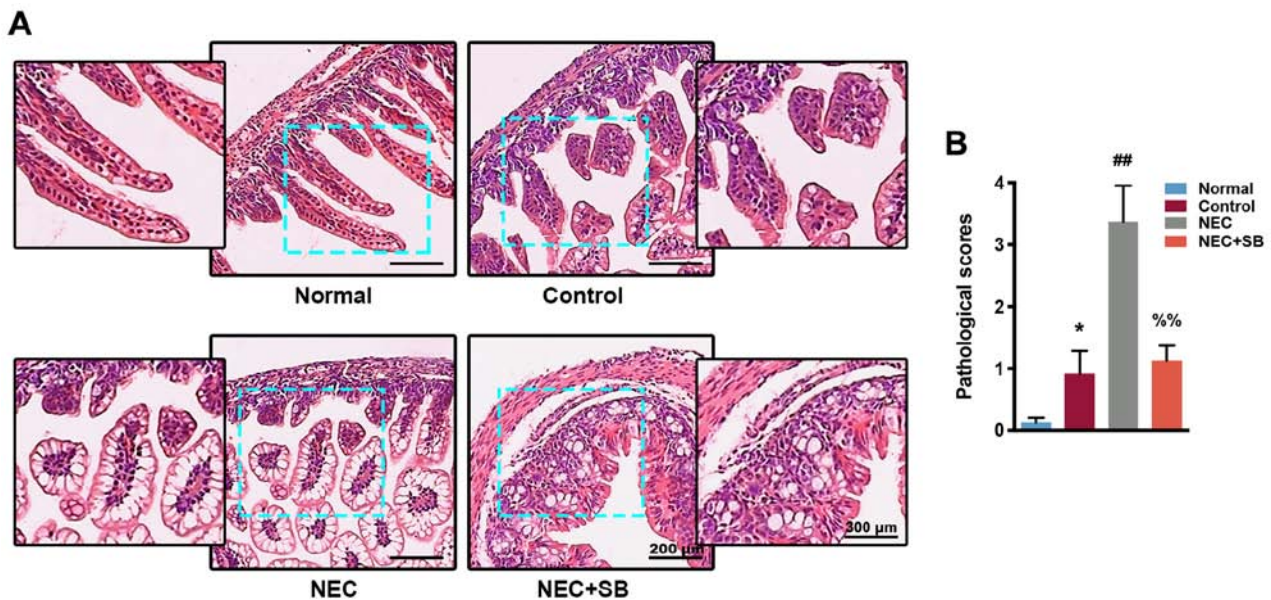


Figure 1. Pathological changes of ileocecal intestinal tissue in neonatal mice. (A) H&E staining revealed pathological changes in ileocecal intestinal tissue in the normal, control, NEC and NEC + SB groups (bar, 200 μm). (B) Pathological scores of ileocecal intestinal tissues in the normal, control, NEC and NEC + SB groups (n=12). Data were compared using Tukey's honest significant difference test. Error bars indicate standard error of mean. \*P<0.05 vs. normal; ##P<0.01 vs. control; %%P<0.01 vs. NEC. H&E, hematoxylin and eosin; NEC, necrotizing enterocolitis; SB, *Saccharomyces boulardii*.

of the intestine in the control (artificial feeding) group was slightly darkened, and the ileum was partially dilated. In the NEC group, the intestine was severely dilated, blackened and congested. In addition, the NEC mice had severe intestinal gas accumulation. The intestine of the NEC + SB neonatal mice was visually relieved, only showing mild edema and only slightly darkened in color, which was significantly improved compared with the NEC group. H&E staining revealed that the ileocecal intestinal tissue of the normal group was intact with continuous epithelium, regular glands and neat villi, and the mucosa, submucosa and lamina propria were free of congestion and edema (Fig. 1A). The pathological score was low in the normal group (Fig. 1B). The ileocecal mucosa, submucosa and lamina propria of the control group exhibited mild congestion and edema, and the glands were disorderly arranged, resulting in an increase in pathological score compared with the normal group (P<0.05). Furthermore, the ileocecum in the NEC group had obvious villi degeneration and edema, disordered gland arrangement, severe congestion, and edema of mucosa, submucosa and lamina propria. The pathological score was significantly higher than that of the control group (P<0.01). The NEC + SB group had a mild edema in the mucosa, submucosa and lamina propria, and the villi were uneven, although there were some arrangements, and the glands were arranged regularly. The pathological score in the NEC + SB group was significantly decreased compared with the NEC group (P<0.01).

*SB induces upregulation of SIRT1 expression in NEC neonatal mice.* The results from immunofluorescence staining indicated that a relatively high level of SIRT1 protein expression was primarily located in the intestinal epithelial cells of the normal group (Fig. 2). Artificial feeding (control) could lead to a decrease in the expression of SIRT1 in the ileocecum of neonatal mice, and there was a further decrease in the level of SIRT1 in the NEC group compared with the control group.

Notably, NEC mice fed with SB could reverse the decrease in SIRT1 and markedly enhance its expression level. These results revealed that SB treatment enhanced SIRT1 protein expression in NEC neonatal mice.

*SB treatment in NEC mice results in an activation of the SIRT1/NF-κB pathway.* To determine whether the SIRT1/NF-κB pathway is involved in the role of SB in NEC mice, western blotting was used to analyze the protein expression of SIRT1 and NF-κB. As revealed in Fig. 3A and B, the expression of cytoplasmic SIRT1 and nuclear NF-κB proteins in the control group was slightly decreased and increased, respectively, compared with the normal group (P<0.05). NEC insult led to a further decrease and increase of SIRT1 and NF-κB (P<0.01). SB treatment in NEC mice reversed the decrease in SIRT1 and the increase in NF-κB, and significantly promoted SIRT1 expression and reduced NF-κB expression (P<0.01).

*SB induces upregulation of SIRT1 and downregulation of NF-κB mRNA levels in neonatal mice with NEC.* Next, the effect of SB on SIRT1, NF-κB, TNF-α and IL-6 mRNA expression in NEC mice was investigated. Similar to the changes in SIRT1 and NF-κB at the protein level, a decrease in SIRT1 and an increase in NF-κB mRNA was observed in the control group compared with the normal group (P<0.05; Fig. 4A and B). NEC insult induced a significant decrease in SIRT1 mRNA and an increase in NF-κB mRNA levels compared with the control group (P<0.01). In addition, the mRNA levels of TNF-α and IL-6 in the NEC group were significantly increased compared with the control group (P<0.01; Fig. 4C and D). SIRT1 mRNA expression was significantly upregulated, and the mRNA levels of the members of the NF-κB pathway were all downregulated in the NEC + SB group compared with the NEC group (P<0.01).

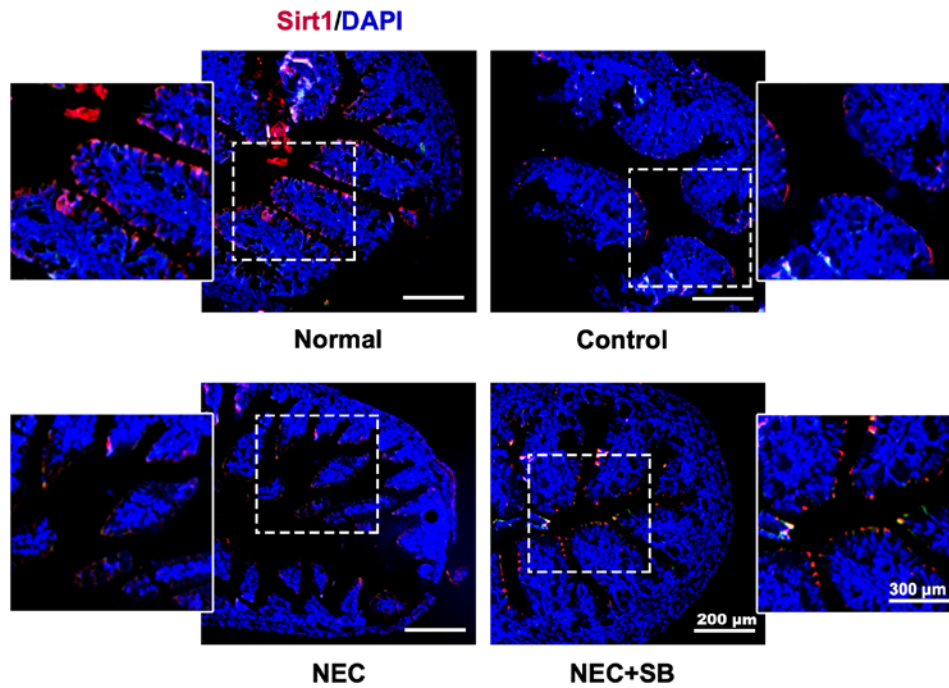


Figure 2. SIRT1 expression and location in ileocecal intestinal tissue of neonatal mice. Immunofluorescence staining was used to detect the expression and localization of SIRT1 protein in ileocecal tissues of mice in the normal, control, NEC and NEC + SB groups (scale bar, 200  $\mu$ m). SIRT1, sirtuin 1; NEC, necrotizing enterocolitis; SB, *Saccharomyces boulardii*.

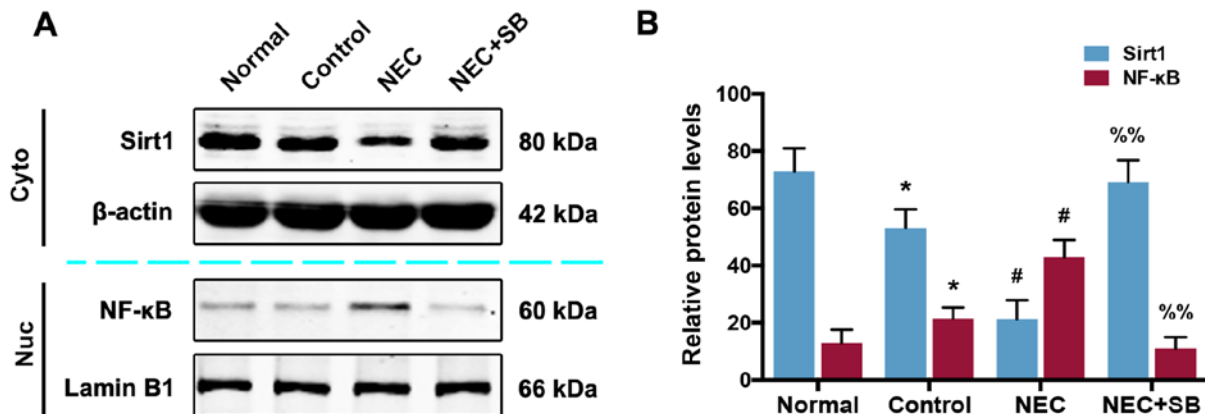


Figure 3. SIRT1 and NF- $\kappa$ B protein expression in ileocecal tissue of neonatal mice. (A) The expression of the cytoplasmic protein SIRT1 and the nuclear protein NF- $\kappa$ B in ileocecal tissues of neonatal mice was determined by western blotting. (B) Western blot data were represented by bar plots (n=7). Data were compared using Tukey's honest significant difference test. Error bars indicate the standard error of mean. \*P<0.05 vs. normal; #P<0.05 vs. control; %%P<0.01 vs. NEC. SIRT1, sirtuin 1; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NEC, necrotizing enterocolitis; Nuc, nucleus; Cyto, cytoplasm; SB, *Saccharomyces boulardii*.

*Intestinal-specific SIRT1 KO (SIRT1<sup>KO</sup>) inhibits the protective effect of SB on NEC neonatal mice.* To further testify the possible function of the SIRT1 pathway in NEC neonatal mice, an intestinal epithelium-specific SIRT1<sup>KO</sup> mouse model was generated, in which the exon 4 of the mouse SIRT1 gene was deleted throughout the length of the intestinal epithelium (20). There was no obvious intestinal damage in SIRT1<sup>KO</sup> mice with breastfeeding or artificial feeding (Fig. 5A). The ileocecal lesions caused by NEC in SIRT1<sup>KO</sup> neonatal mice were significantly increased compared with the control (co-housed paired Flox) group, and SB treatment did not reverse this pathological damage. Furthermore, the pathological score of SIRT1<sup>KO</sup> with artificial feeding was increased compared with SIRT1<sup>KO</sup> with breastfeeding (P<0.05). The score of SIRT1<sup>KO</sup> in the

NEC + SB group was also significantly increased compared with the control group (P<0.01). In addition, both the SIRT1 protein (Fig. 5B and C) and mRNA (Fig. 5D) expression in the SIRT1<sup>KO</sup> NEC + SB group were significantly decreased compared with the control group (P<0.01). Unexpectedly, the NF- $\kappa$ B protein (Fig. 5B and C) and mRNA (Fig. 5E) levels, and the mRNA levels of its downstream partners TNF- $\alpha$  and IL-6 (Fig. 5F and G) were all significantly increased in the SIRT1<sup>KO</sup> group compared with the control group (P<0.01). All these results indicated that knockdown of SIRT1 suppressed the effect of SB in NEC mice.

*Protective role of SB in NEC neonatal mice is also associated with gut microbiota regulation.* To evaluate whether



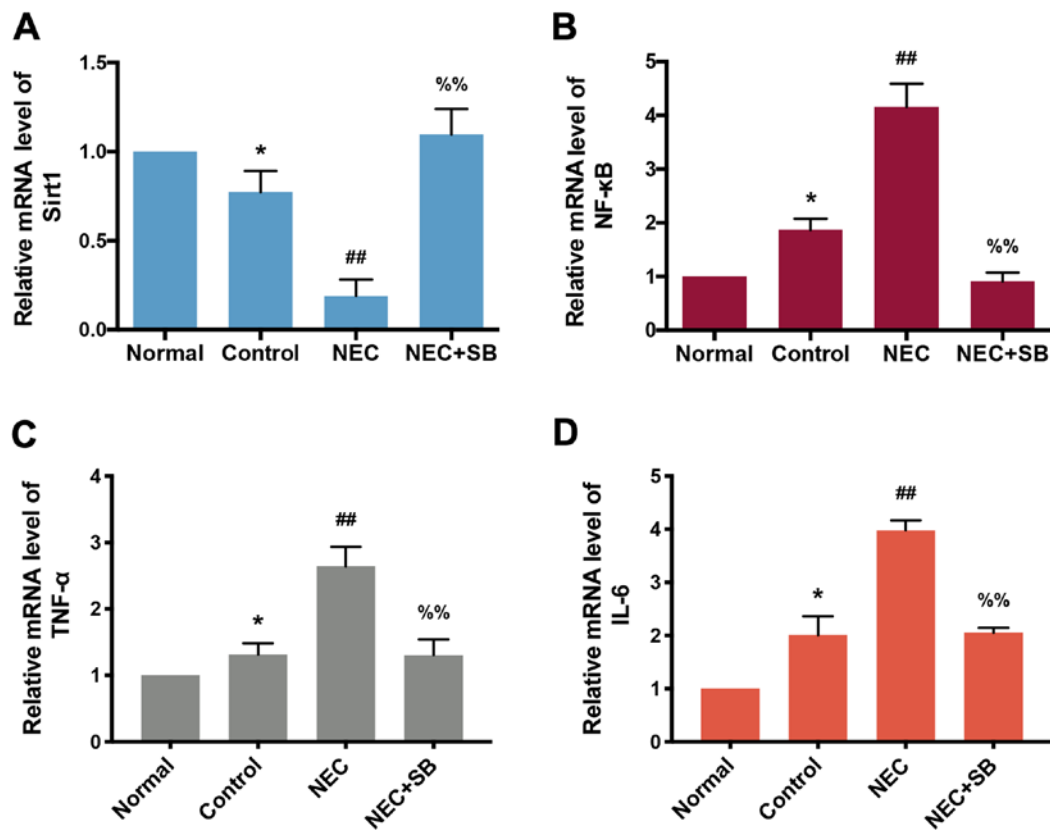


Figure 4. SIRT1 and NF- $\kappa$ B mRNA levels in ileocecal intestinal tissue of neonatal mice. (A) SIRT1 mRNA expression in the normal, control, NEC and NEC + SB groups was determined by RT-qPCR (n=7). (B) mRNA levels of NF- $\kappa$ B in the normal, control, NEC and NEC + SB groups were analyzed by RT-qPCR (n=7). (C and D) The mRNA levels of (C) TNF- $\alpha$  and (D) IL-6 in the normal, control, NEC and NEC + SB groups were analyzed by RT-qPCR (n=7). Data were compared using Tukey's honest significant difference test. Error bars indicate the standard error of mean. \*P<0.05 vs. normal; ##P<0.01 vs. control; %%P<0.01 vs. NEC. SIRT1, sirtuin 1; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NEC, necrotizing enterocolitis; SB, *Saccharomyces boulardii*; RT-qPCR, reverse transcription-quantitative PCR; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6.

SB-induced alterations in NEC mice is associated with the regulation of the gut microbial ecosystem, total fecal microbiota profiles of neonatal mice were analyzed via 16S rRNA amplicon sequencing. Feces were collected, and the proportions of microbiota were evaluated by qPCR. As revealed in Fig. 6A, the abundance of all gut microbial composition except Lact, including Erec, Clept, Bact, MIB and SFB were decreased in the control group compared with the normal group (P<0.05). Furthermore, NEC insult induced a significant decrease in gut microbial abundance compared with the control group (P<0.01 in Erec, Clept and Lact; P<0.05 in Bact, MIB and SFB). SB treatment significantly upregulated the abundance of gut microbiota in NEC mice compared with the NEC group (P<0.01 in Erec, Clept, Lact and Bact; P<0.05 in MIB and SFB). Notably, the gut microbial abundance in the SIRT1<sup>KO</sup> NEC + SB group was significantly decreased compared with the Flox NEC + SB group (P<0.01; P<0.05 in Lact; Fig. 6B). These results revealed that the modulation of gut microbiota was also involved in the role of SB in NEC neonatal mice.

## Discussion

NEC is one of the most common and devastating gastrointestinal emergencies in markedly low birth weight (<1,500 g) infants in neonatal intensive care units, with reported

mortality rates between 15 and 30% (1,26). NEC is a multifactorial disease, and is a largely unpredictable disease in newborns, the etiology of which remains unclear despite the advances in research (1). Multiple factors are reported to be responsible for the pathogenesis of NEC, including immaturity of the gut, hypoxia-ischemia, artificial (namely formula) feeding and gut microbial dysbiosis, which play a role in inducing an inflammatory response in the gut (27). Inflammatory mediators such as NF- $\kappa$ B are activated to produce an inflammatory cascade response, contributing to mucosal damage (28). This inflammatory cascade induces the activation of neutrophils, increases the permeability of the vasculature and releases reactive oxygen species, leading to vasoconstriction with ischemic-reperfusion injury (28). The mucosal barriers in the gut continue to break down, resulting in a severe NEC, which could lead to sepsis or even death (29).

The yeast SB is a known 'generally regarded as safe' microorganism with probiotic activity against various types of microbial pathogens in the intestine (30). According to previous studies, SB treatment in mice induces an immunomodulatory effect, and leads to an increased level of secretory IgA and serum IgG (31,32), as well as serum IgM (33). These immunological roles, along with other probiotic features of SB, such as bile and acid resistance, and an optimum growth at 37°C, make SB a potential therapy with oral delivery in

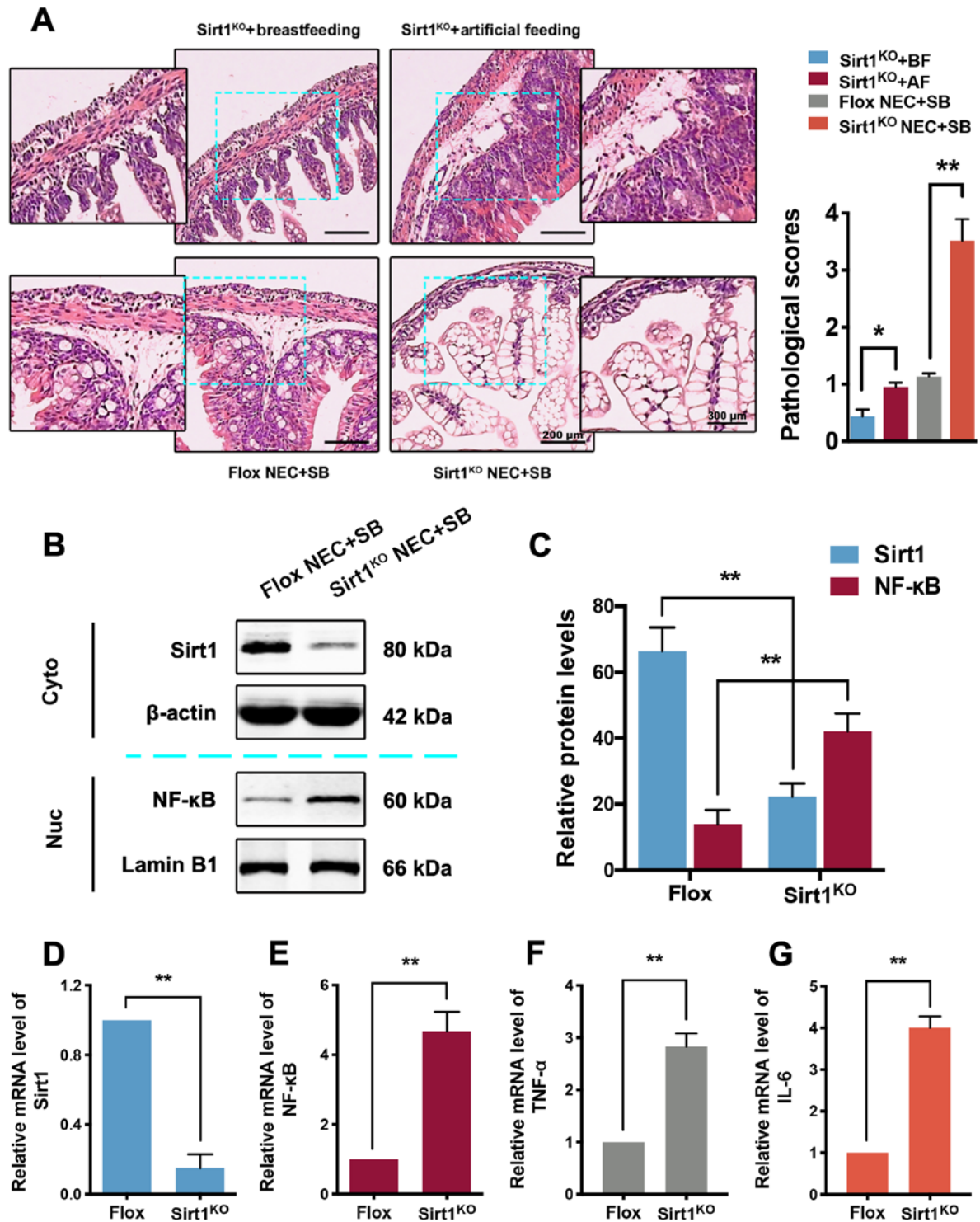


Figure 5. Pathological changes, SIRT1 and NF- $\kappa$ B expression in the ileocecum of the intestinal epithelium of SIRT1<sup>KO</sup> mice. (A) Pathological changes (bar, 200  $\mu$ m) and scores (n=3) in the ileocecum of SIRT1<sup>KO</sup> mice with breastfeeding, SIRT1<sup>KO</sup> mice with artificial feeding, control + NEC + SB mice and SIRT1<sup>KO</sup> + NEC + SB mice (n=7). \*P<0.05, \*\*P<0.01 vs. indicated group. (B) The protein expression of SIRT1 and NF- $\kappa$ B in SIRT1<sup>KO</sup> and control mice was determined by western blotting. (C) Western blot data are presented by bar plots (n=3). The mRNA expression of (D) SIRT1, (E) NF- $\kappa$ B, (F) TNF- $\alpha$  and (G) IL-6 in SIRT1<sup>KO</sup> and control mice was analyzed by RT-qPCR (n=3). \*\*P<0.01 vs. indicated group. Data were compared using the Tukey's honest significant difference test. Error bars indicate standard error of mean. SIRT1, sirtuin 1; NF- $\kappa$ B, nuclear factor- $\kappa$ B; KO, knockout; NEC, necrotizing enterocolitis; SB, *Saccharomyces boulardii*; BF, breastfeeding; AF, artificial feeding; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6.

intestinal diseases (30,34). However, whether SB has an effect on the inflammatory response and microbial dysbiosis in the pathogenesis of NEC mice remains to be explored.

SIRT1 is essential for cell survival, differentiation, senescence and metabolism due to its role on anti-inflammation and

antioxidation (35). SIRT1 deacetylates NF- $\kappa$ B p65 subunit at Lys310, which results in inhibition of NF- $\kappa$ B activity and transcriptional silence of pro-inflammatory genes, such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (36,37). The function of SIRT1 and NF- $\kappa$ B in the role of SB in NEC mice warrants further study.

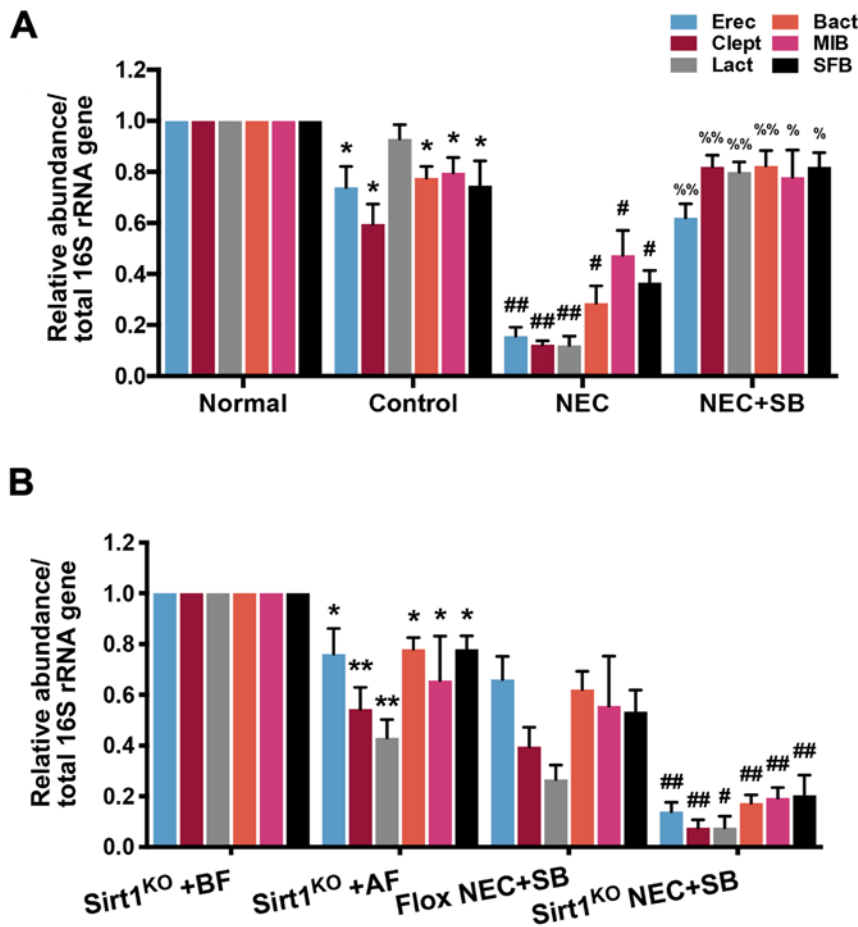


Figure 6. Abundance of gut microbial composition in neonatal mice. (A) The relative abundance of gut microbiota in normal, control, NEC and NEC + SB mice was detected by qPCR (n=7). (B) The relative abundance of gut microbiota in SIRT1<sup>KO</sup> mice with BF, SIRT1<sup>KO</sup> mice with AF, SIRT1<sup>KO</sup> NEC + SB mice and Flox NEC + SB mice was determined by qPCR (n=3). \*P<0.05, \*\*P<0.01 vs. normal or SIRT1<sup>KO</sup> + BF; #P<0.05, ##P<0.01 vs. control or Flox NEC + SB; %P<0.05, %%P<0.01 vs. NEC. Data were compared using Tukey's honest significant difference test. Error bars indicate standard error of mean. NEC, necrotizing enterocolitis; SB, *Saccharomyces boulardii*; qPCR, quantitative PCR; SIRT1, sirtuin 1; KO, knockout; BF, breastfeeding; Erec, *Eubacterium rectale*/*Clostridium coccoides*; Clept, *Clostridium leptum*; Lact, *Lactobacillus* sp.; Bact, *bacteroides* sp.; MIB, mouse intestinal *Bacteroides*; SFB, segmented filamentous bacteria.

The present study demonstrated that SB relieved the NEC insult in neonatal mice through the SIRT1/NF-κB pathway and gut microbial regulation. SIRT1 deficiency in the intestinal epithelium results in increased pathological damage and NF-κB expression, which in turn leads to a reduction in gut microbial composition. The pathological injury and score in the control group were higher than those of the normal group, and a further increase was observed in the NEC group. Treatment with SB in the NEC group significantly reduced both the pathological injury and score. Furthermore, the expression and location of SIRT1 protein was verified by immunofluorescence staining, and a relatively high expression of SIRT1 protein expression, which was primarily located in the intestinal epithelial cells of the normal group, was determined. NEC insult induced a marked decrease in SIRT1 expression, which was inhibited by SB treatment. Furthermore, the results from western blotting and RT-qPCR revealed that the protein and mRNA expression levels of SIRT1 were decreased, and the expression levels of NF-κB and its downstream cytokines were all increased in the normal group compared with the control group. A further decrease in SIRT1 and an increase in the expression of molecules of the NF-κB pathway was

observed in NEC neonatal mice. SB treatment significantly relieved the NEC role on the SIRT1/NF-κB pathway activation. In addition, intestinal epithelium-specific SIRT1<sup>KO</sup> abolished the effects of SB in NEC neonatal mice, including a decrease in pathological damage and score, inhibition of SIRT1/NF-κB pathway activation, and changes in the mRNA expression of SIRT1 and NF-κB. Finally, the abundance of gut microbial composition, including Erec, Clept, Bact, MIB and SFB (but not Lact) was decreased in the control group compared with the normal group. NEC insult further decreased the abundance of microbiota (including Lact) compared with the control group. SB treatment reversed the decrease in all gut microbiota in NEC neonatal mice. As anticipated, the effect of SB on gut microbiota modulation was abolished in SIRT1<sup>KO</sup> neonatal mice. These results indicated that the abundance of gut microbial composition was modulated by SIRT1. Notably, there was a mild gut dysbiosis in SIRT1<sup>KO</sup> mice with artificial feeding (without NEC) compared with SIRT1<sup>KO</sup> mice with breastfeeding. The present study hypothesized that the dysbiosis of intestinal microbiota is more likely to be the cause rather than the consequence of NEC, as there is still gut dysbiosis in the absence of NEC. The



possible mechanisms of the SB effect on SB expression may be associated with a direct enhancement in SIRT1 gene transcription or with indirect regulation of specific microRNAs and/or long noncoding RNAs (lncRNAs). The main limitations of this study are the lack of exploration of the specific mechanisms of the roles of SB on gut microbiota regulation and the absence of relative human tissue samples. In addition, since numerous other signaling pathways (including TLRs, JNK/STAT and cAMP) also affect gut inflammation, further studies to verify the association between SB and these pathways are required. Furthermore, as NEC can be divided into three stages (namely I, II and III), stages II and III were included in the present study; however, the present study did not differentiate between the therapeutic effect of SB in stages II and III.

Collectively, the present study provides a new insight and further understanding of the mechanisms of the protective role of SB on NEC. Furthermore, the present results provide a theoretical basis for SB as a preventive and therapeutic agent for NEC in the future.

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### Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

KZ designed the research, performed the experiments, analyzed the data and wrote the paper. XZ designed and performed experiments and analyzed the data. AL performed and analyzed experiments. SF provided technical support and substantial contributions to conception and design. JZ directed the study, managed the project, interpreted the data and co-wrote the paper. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee on Animal Care of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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