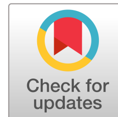

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***Lactobacillus* protects against *S. typhimurium*-induced intestinal inflammation by determining the fate of epithelial proliferation and differentiation**

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Short title: *Lactobacillus* alleviates mucosal damage

Keywords: *Lactobacillus*; *S. typhimurium*; intestinal epithelial; proliferation; intestinal stem cells

Abbreviations: IBD: Inflammatory bowel disease； ISCs: Intestinal stem cells; SAL: Salmonella (*S. typhimurium*); LAC: *Lactobacillus* (*L. acidophilus*); SPF: Specific Pathogen

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Free; PBS: Phosphate buffer saline; EDTA: Ethylenediaminetetraacetic acid; EGF: Epidermal Growth Factor; CFU: Colony-forming unit; HE: Hematoxylin-eosin; MLN: Mesenteric lymph nodes; TNF-α: Tumor necrosis factor α; UEA-1: Ulex europaeus agglutinin-1; PCNA: Proliferating cell nuclear antigen; DCLK-1: Doublecortin-like kinase 1

Abstract:

Scope: The influence of the intestinal microbiota, such as *Lactobacillus*, on the intestinal mucosa, particularly intestinal stem cells, remains incompletely understood. In this study, mice and intestinal organoids were used to explore the regulatory effect of *Lactobacillus* on the proliferation and differentiation of intestinal epithelial cells.

Methods and results: This study demonstrated that *S. typhimurium* caused intestinal epithelial damage and affected growth of intestinal organoids. *S. typhimurium* also colonized the intestine and then caused pathological changes to the intestinal epithelium, intestinal inflammation, and even death. However, *L. acidophilus* alleviated damage to intestinal organoids, increased the survival ratio of mice infected with *S. typhimurium*, and reduced TNF-α secretion. Moreover, *L. acidophilus* affected the differentiation of epithelial cells through inhibition of the excessive expansion of goblet cells and Paneth cells induced by *S. typhimurium* to avoid over-exhaustion. Finally, we also demonstrated that *L. acidophilus* ameliorated overactivation of Wnt/β-catenin pathway by *Salmonella*, depending on the contact with TLR2, to affect the proliferation of the intestinal epithelium.

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Conclusions: This study demonstrated that *L. acidophilus* protects the intestinal mucosa against *S. typhimurium* infection through not only the inhibition of pathogen invasion but also determination of the fate of the intestinal epithelium.

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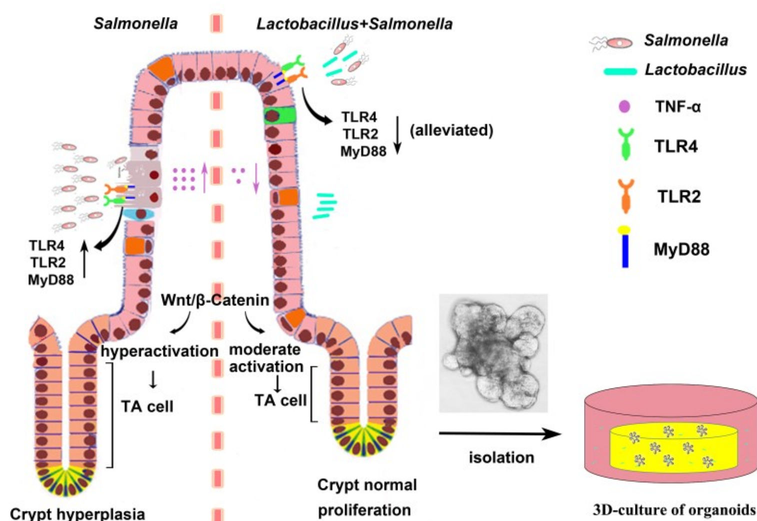

TOC Graphic description

This study revealed that *L. acidophilus* ATCC 4356 modulated Wnt/β-catenin pathway through TLR2 and affected proliferation of intestinal epithelial, as well as the differentiation of Paneth cells and goblet cells, to alleviate intestinal inflammation and crypt hyperplasia induced by *S. typhimurium* infection. The presentative results puts an insight on clinical treatment that besides antibiotics, *L. acidophilus* is also effective in curing diarrhea and improving intestinal inflammation.

1. Introduction

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The intestinal mucosa is continuously in contact with various food particles and the intestinal microbiota, including enteropathogens, which might cause intestinal inflammation [1]. Inflammatory bowel disease (IBD) refers to a set of inflammatory conditions of the colon and small intestine that can be very painful and disruptive [2, 3]. Although the cause of IBD is complicated and has not been fully elucidated, a recent study demonstrated that the intestinal microbiota affects the proliferation and differentiation of intestinal stem cells (ISCs) and is closely related to IBD [4]. Salmonella enterica serovar typhimurium *S. typhimurium* is a gram-negative, facultative, intracellular anaerobe that causes severe inflammation of the intestinal mucosa to result in gastroenteritis, including IBD [5, 6]. The choice of IBD therapy, such as anti-inflammatory drugs, immune system suppressors, and antibiotics, always causes side effects on human health [7, 8].

The proliferation of the intestinal epithelium due to the presence of intestinal stem cells (ISCs) constitutes the basis for the maintenance of the intestinal mucosal barrier against pathogen invasion and intestinal inflammation [9, 10]. The intestinal microbiota is continuously in contact with the epithelium and affects niches of ISCs to influence the proliferation and differentiation of the epithelium [11-13]. Moreover, Wnt signaling directly controls cell fate, such as proliferation, differentiation, and apoptosis [14]. Dysregulation of the Wnt signaling pathway has been shown to be closely related to multiple tumorigenesis [15, 16]. Previous studies have demonstrated that *Lactobacillus* can effectively prevent pathogen invasion and protect the integrity of the intestinal mucosal barrier [17-19]. However, the probiotic role of *Lactobacillus* mainly focuses on the induction of a low pH value, antimicrobial peptide secretion and tight junction maintenance [20, 21].

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Traditional studies of *Lactobacillus* that focused on the intestinal mucosal barrier with single cell lines, such as Caco-2 cell line, could not clearly mimic the *in vivo* intestinal conditions [22-24]. L. acidophilus is one of the most commonly used probiotics in the food supply [25]. Our previous study demonstrated that *L. acidophilus* ATCC4356 and its S-layer protein effectively inhibit the adhesion of *S. typhimurium* to Caco-2 cells through modulation of the MAPK signaling pathway [26]. Intestinal organoids containing ISCs could form intestinal villi and crypts and differentiate into absorptive cells, goblet cells, enteroendocrine cells, Paneth cells and tuft cells; as a result, these organoids constitute a promising model for exploring the interaction between the intestinal microbiota and the intestinal mucosa [27, 28]. In this study, we further hypothesized that *L. acidophilus* can effectively maintain intestinal homeostasis and prevent intestinal inflammation and crypt hyperplasia induced by *S. typhimurium* infection through modulation of the proliferation and differentiation of the intestinal epithelium and investigated this hypothesis using intestinal organoids as a model as well as mice experiments.

2. Materials and Methods

2.1. Bacterial strain culture

The bacterial strains used in this study included *S. typhimurium* strain SL1344 and *L. acidophilus* ATCC4356. *S. typhimurium* strain SL1344 was grown overnight at 37°C with shaking (200 rpm) in LB broth supplemented with 100 g/ml streptomycin. *Lactobacillus* ATCC4356 was grown on MRS agar at 37°C in an anaerobic CO_2 enriched atmosphere.

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2.2. Isolation and culture of intestinal organoids

Intestinal organoids were isolated from small intestines of 4-week-old SPF C57BL/6 mice. The dissected intestine samples were cleaned with ice-cold PBS and cut into pieces. After the supernatant became clear, EDTA buffer (2 mM) was added, and the mixture was then digested at 4°C for 30 min and shaken for 1 min. The intestinal crypt suspension was filtered through a 70-μm cell strainer and centrifuged at 800g for 5 min at 4°C, and the supernatant was gently poured off. The intestinal crypts were resuspended in Matrigel (BD Biosciences) and then seeded onto tissue culture plates. The Matrigel suspension was allowed to polymerize at 37°C for 30 min before the addition of fresh complete medium (Advanced DMEM/F12 (Gibco) supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, 10 mM HEPES, N-2, and B-27, 50 ng/mL EGF, 100 ng/ml Noggin, and 500 ng/ml R-spondin1). The culture medium and growth factors were replaced every 3-4 days. For the passage of intestinal organoids, the plates were rinsed with ice-cold PBS and pipetted to break the organoids in Matrigel. The organoids were resuspended in 1 ml of cold PBS, collected in tubes and centrifuged at 4°C and 150 g for 10 min. The crypts were resuspended in Matrigel with growth factors and cultured prior to the following steps.

2.3. *Lactobacillus* pretreatment and *S. typhimurium* infection of organoids

The organoids were cultured with bacteria as previously described [29, 30]. After centrifugation at 400 × g and 4°C for 15 min, the collected bacteria were washed three times with PBS and subsequently resuspended with PBS for the treatment of intestinal organoids. Intestinal organoids were seeded in a 24-well plate after passage and treated with *L.*

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acidophilus (10^8 CFU) in antibiotic-free culture for 12 h. Subsequently, to mimic the process of infection *in vivo*, the Matrigel was removed with cold PBS, and the budding part of the crypt was then pipetted and broken up to expose the gap and thus allow *S. typhimurium* to invade the apical surface of the organoids as much as possible. After infection with *S. typhimurium* (10^7 CFU) for 1 h, the intestinal organoids were reseeded with Matrigel on the 24-well plate and recovered with media containing gentamicin (100 μg/ml, Sigma-Aldrich) for 24 h to kill extracellular bacteria. To ensure bacteria could get touch with enteric cavity, we added Lactobacillus and *S. typhimurium* when passaged the intestinal organoids. The growth status and morphology of the intestinal organoids were observed under a light microscope.

In order to verify the effect of *Lactobacillus* on Wnt/β-catenin signal pathway, budding organoid were also pretreated with *Lactobacillus* combined with or without toll like receptor 2 inhibitor CU-CUPT22 (0.02%, Selleck, USA) for 12h and then the intestinal organoids were infected by *S. typhimurium* with or without toll like receptor 4 inhibitor atractylenolide I (1%, Selleck, USA) for 1h[31, 32].

In order to inhibit or activate the Notch signal pathway to regulatec the differentiation of goblet cells ,10 μmol/L DAPT (Selleck, USA) or 5μg/ml Dll4 (Abcam, Britain) was added to the intestinal organoids supernatant.

2.4. Animal treatments

Specific-pathogen-free C57BL/6 mice were purchased from Yangzhong University and randomly divided into four groups. The mice were orally administered sterile PBS (Control

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group), *S. typhimurium* SL134 (10^7 colony-forming unit (CFU), Sal group) or *Lactobacillus* ATCC4356 (10^8 CFU, Lac group). The fourth group of mice was pretreated with *Lactobacillus* for 1 month and then infected with *S. typhimurium* SL1344 (Lac+Sal group) (Fig. 3A). The survival of the various mice was recorded, and feces were collected for CFU analysis. The mice were sacrificed through exposure to carbon dioxide, and tissue samples were collected for further analysis. The animal protocol was approved by the University of Nanjing Agriculture University Committee on Animal Resources Committee (permission number: 201718095).

2.5. Detection of *Lactobacillus* ATCC4356 strain in the small intestine

Bacterial DNA from the digested jejunum of mice was extracted using a stool DNA kit (OMEGA, USA) according to the manufacturer's instructions. Quantitative real-time PCRs were performed in duplicate using the Applied Biosystems 7500 real-time PCR system with a SYBR master kit. The primers used to quantify the *Lactobacillus* ATCC4356 strain are listed in Table 1. The reaction mixture consisted of 10 μl of SYBR green I master, 2 μl of DNA sample, and the various primer in a final volume of 20 μl. The thermal cycling conditions were 40 cycles of 15 s at 95°C and 34 s at 60°C followed by cooling at 4°C, and these were implemented using an Applied Biosystems 7500 real-time PCR system. The standard curve was constructed from serial dilutions of DNA solutions extracted from each bacterial group using a DNA ASSAY kit (Tiangen, China). Real-time monitoring was achieved by measuring the fluorescence at the end of the elongation phase.

2.6. Morphology of intestinal tissue

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For the observation of intestinal pathological changes, the intestine tissue was fixed in 4% paraformaldehyde for 24 h, dehydrated in alcohol (for 1 h each in 70, 80, 90, and 100%) and xylene for 40 s and embedded into paraffin. The paraffin block was sliced at 5-μm thickness and stained with hematoxylin-eosin (HE).

2.7. *S. typhimurium* colonization and secretion of proinflammatory cytokines in mice

Feces from the various groups of mice were harvested and placed into tubes with sterile PBS. MLNs were ground, filtered through a cell strainer to obtain the supernatant and diluted in LB. The bacteria suspension was plated on agar plates with 100 g/ml streptomycin and incubated overnight at 37°C, and the CFUs were quantified. The production of proinflammatory cytokines (TNF-α) was measured with an ELISA kit (Boster, China) according to the manufacturer's recommended protocol.

2.8. Quantitative RT-qPCR

Tissue samples from mice and treated organoids were harvested, and total RNA was extracted using RNAiso Plus (Takara, Japan). Reverse transcription of the RNA was performed with the primers listed in Table 1. Two microliters of template RNA was reacted with Taq-Man PCR Master Mix in a final volume of 20 μl (Takara, Japan). The thermal cycling conditions, which were implemented with an Applied Biosystems 7500 real-time PCR system, were 5 min at 95°C followed by 40 cycles of 15 s at 95°C and 34 s at 60°C.

2.9. Immunofluorescence

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For *in vitro* imaging, intestinal organoids derived from mice were embedded in Matrigel on glass chamber slides. The cells were fixed for 15 min at room temperature using 4% paraformaldehyde and rinsed three times with PBS. The cell slides were stained as described previously. The organoids were visualized using lysozyme and Ulex europaeus agglutinin-1 (UEA-1) to indicate Paneth and goblet cells, respectively.

Intestinal tissues slides were deparaffinized and rehydrated. To achieve enhanced immunoreactivity, antigen retrieval was performed by incubating the slides in 10 mM sodium citrate for 30 min at 80°C. The slides were then cooled to room temperature, washed in PBS for 3 min and blocked in 0.4% Triton-100 for 20 min to allow permeation of the primary antibody to the tissue. The slides were then blocked and then incubated for 2 h with 5% BSA, overnight at 4°C with the primary antibody (rabbit anti-proliferating cell nuclear antigen (PCNA), rabbit anti-lysozyme, rabbit anti-doublecortin-like kinase 1 (DCLK-1), and rabbit anti-β-catenin) diluted 1:1000 in 0.01 M PBS and then for 1 h with Alexa Flour 488- or 594-conjugated secondary antibodies. DAPI (Life Technologies, USA) was used to counterstain nuclei, and the resulting staining was observed with a Zeiss scanning confocal microscope.

2.10. Western blot

Tissue samples from mice were lysed in RIPA buffer containing a protease inhibitor cocktail (Thermo Scientific). Protein concentrations were detected using a BCA protein quantification kit (Thermo Scientific). Equal amounts of protein were separated by SDS-PAGE and electrophoretically transferred onto PVDF membranes (Millipore, China). The membrane

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was then blocked with 5% nonfat milk in TBS containing 0.1% Tween-20 and then probed with rabbit anti-β-catenin (Abcam, 1:1000) and rabbit anti-GAPDH (Bioworld, 1:1000) for normalization. The membranes were then washed and incubated with goat and mouse anti-rabbit secondary antibodies (Vazyme, 1:5000). Signals were detected using a SuperSignal West Pico kit (Thermo Scientific) and analyzed with an Image Reader LAS-4000 imaging system.

2.11. Flow cytometry

The intestinal organoids were digested with 0.25% pancreatin (Gibco) for 5 mins to obtain single cell. Cell apoptosis was tested by cell apoptosis detection kit (Elabscience). Samples and data were collected and analyzed with a BD-related device and software (FACSCalibur and Cell Quest-Pro).

2.12 Statistical analysis

The results are expressed as the means ± SDs. One-way ANOVA was employed to determine the significance of the differences among multiple groups, and a t-test was employed to determine the significance of the differences between two groups. The significance levels are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

3. Results

3.1. *L. acidophilus* protected the intestinal mucosa of mice against *S. typhimurium* infection

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According to the study protocol (Fig. 1A), mice infected with *S. typhimurium* by oral gavage died started form 2 days post infection and exhibited serious pathological bleeding in the jejunum lumen and muscular layer and even intestinal villi shedding (Fig. 1C and 1E). Moreover, colonization of *Lactobacillus* ATCC4356 also reached a peak in 36h (Fig. 1B). We also found that pretreatment with *L. acidophilus* ATCC4356 reduced the burden of *S. typhimurium* in feces and MLNs (Fig. 1D). The severe damage to the intestinal morphology might be attributed to increased secretion of proinflammatory cytokines (TNF-α) (Fig. 1F). However, *L. acidophilus* pretreatment significantly reduced the death rate and secretion of proinflammatory cytokines. The protective effect of *L. acidophilus* on the intestine was also demonstrated on the maintenance of the intestinal morphology (Fig. 1C). The intestinal inflammation and epithelial damage caused by *S. typhimurium* could be ameliorated by *L. acidophilus* by diminishing damage to the intestinal villi.

3.2. Regulatory effect of *L. acidophilus* on intestinal secretory cell hyperplasia

In this study, we found that the oral administration of *Lactobacillus* did not affect the numbers of goblet cells (UEA-1) (Fig. 2A), Paneth cells (lysozyme) (Fig. 2B) and tuft cells (Dclk1) (Fig. 2C) in the mice intestine under physiological conditions. However, *Salmonella* infection induced significant expansion of the number of secretory cells in the host to protect the mucosal barrier and defend against *Salmonella* invasion. Surprisingly, *L. acidophilus* ATCC4356 increased the levels of goblet cells, Paneth cells and tuft cells to a reasonable level to avoid over-exhaustion of intestinal secretory cells.

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3.3. Modulation of the Wnt/β-catenin pathway by *L. acidophilus* affects intestinal proliferation

Compared with the control group, *Salmonella* infection significantly increased the number of PCNA$^+$ epithelial cells (Fig. 3A), which might be attributed to overactivation of the Wnt/β-catenin pathway by the overexpression of β-catenin (Fig. 3B). The overexpression of β-catenin was further confirmed by immunofluorescence staining of β-catenin$^+$ cells in the intestine (Fig. 3C). The oral administration of *Lactobacillus* did not affect the expression of TLR2 and TLR4 to maintain the homeostasis of the intestinal barrier in mice. Moreover, pretreatment with *Lactobacillus* sharply limited the activation of TLR2 and TLR4 and reduced MyD88 expression during *Salmonella* infection (Fig. 3D). Moreover, a similar stimulatory effect in the expression of Wnt3 was also observed (Fig. 3E). Under normal physiological conditions, *L. acidophilus* ATCC4356 did not affect β-catenin expression and intestinal proliferation. Interestingly, the *S. typhimurium*-induced increases in the number of PCNA$^+$ epithelial cells and β-catenin expression were alleviated in mice pretreated with *L. acidophilus*. In the meanwhile, the expression of TLR2, TLR4 and MyD88 followed the same trend with the β-catenin expression. Furthermore, *L. acidophilus* rescued the loss of Lgr5$^+$ stem cells after *Salmonella* infection to support the regeneration of the intestinal mucosa barrier (Fig. 3F).

3.4. *L. acidophilus* alleviated damage to intestinal organoids infected with *Salmonella*

S. typhimurium infection caused the disappearance and disintegration of villi in intestinal organoids starting 1 h post infection and attenuated the rate of organoid formation. Most

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organoids were dead within 24 h after infection with *Salmonella* (Fig. 4A). However, this disruption of organoids in response to *Salmonella* infection could be significantly alleviated by pretreatment with *L. acidophilus* ATCC4356 (Fig. 4B), and similar results were obtained for the formation of intestinal organoids (Fig. 4C). The protective effect of *L. acidophilus* on the morphology and growth of intestinal organoids might be attributed to the reduction of TNF-α secretion by *Lactobacillus* (Fig. 4D).

3.5. Modulatory effect of *L. acidophilus* on intestinal secretory cells in organoids through Wnt/β-catenin pathway

The intestinal organoids enhanced the number of goblet cells stained with UEA (Fig. 5A) and increased Muc2 mRNA expression (Fig. 5B) to defend against *S. typhimurium* invasion. A similar stimulatory effect after *Salmonella* invasion was also observed in terms of the number of lysozyme$^+$ cells (Fig. 5C) and Reg3g and Defa1 (Fig. 5D) expression, which indicated that *Salmonella* infection induced defense of the host mucosa. However, pretreatment with *Lactobacillus* could also maintain high expression levels of Reg3g, Defa1 and Muc2 to enhance the mucosal barrier, and this stimulatory effect of *Lactobacillus* was further strengthened after *S. typhimurium* invasion. Furthermore, pretreatment with *Lactobacillus* restricted the over-activation of TLR2 and TLR4 and induced a moderate increase of MyD88 expression during *Salmonella* infection in intestinal organoids (Fig. 5E).

Salmonella infection also induced the hyperexpression of Wnt3 and relative loss of Lgr5, which was reversed with addition of TLR4 inhibitor atractylenolide I (Fig. 5F and 5G). We

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also found that *Lactobacillus* could also significantly increased the Lgr5 expression, while this stimulation was disappeared with the addition of TLR2 inhibitor CU-CUPT22 (Fig. 5G). Moreover, *Lactobacillus* could alleviate the high expression of Wnt induced by *Salmonella* infection (Fig. 5F).

Lactobacillus also alleviated damage to intestinal organoids and ratio of apoptotic cells caused by TNF-α, while the treatment of DAPT can enhance the therapeutic effect of *Lactobacillus*. But Dll4 showed exactly the opposite effect (Fig. 6A-B). DAPT also increased the expression levels of Muc2, Defa1 and Reg3g to protect (Fig. 6C).

4. Discussion

Clinical treatment with probiotic bacteria has been found to efficiency protect against pathogens invasion or intestinal inflammation [33-35]. However, the mechanism underlying the anti-inflammatory effect of probiotics has not yet been fully understood due in part to the complex intestine environment *in vivo*. This study demonstrated the *L. acidophilus* ATCC4356 has protective effects on the maintenance of the normal morphology of intestine, which might be relevant to a decrease in the level of proinflammatory cytokines. Consistent with the *in vivo* results, *L. acidophilus* ATCC4356 also suppressed intestinal inflammation caused by *Salmonella* challenge in intestinal organoids. In this study, we found that *L. acidophilus* ATCC4356 effectively reduced the *S. typhimurium* SL1344 loads in feces and MLNs, whereas *Salmonella* led to mass colonization. These findings are in agreement with recent surveys that showed that *Salmonella* gains a growth advantage over the intestinal

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microbiota in an inflammatory environment [36]. *S. typhimurium* was found that increased the secretion of inflammatory cytokines (TNF-α), intestinal pathological changes and mortality in mice, which is consistent with previous reports[37, 38]. Our previous study deduced that surface-layer (S-layer) proteins from *L. acidophilus* ATCC4356 inhibited caspase-3 activity to protect against *Salmonella*-induced apoptosis in Caco-2 cells [39]. Here, we further demonstrated that *L. acidophilus* reduced TNF-α secretion to ameliorate intestinal inflammation, and this finding was verified by maintenance of the normal intestinal morphology and an increased survival ratio.

Goblet, Paneth and tuft cells are intestinal antimicrobial peptide (AMP)-secreting cells, which are important for protecting the integrity of the intestinal mucosal barrier [40, 41]. Intestinal goblet cells in the intestinal tract secrete mucus and trefoil factor (TFF) 3 to form the first line of defense in the mucosa; Paneth cells are an important source of antimicrobial peptides in the intestine; and tuft cells regulate type 2 immune responses through the secretion of IL-25 to protect the mucosa against helminth infection [42, 43]. *S. typhimurium* infection stimulates the hyperplasia of goblet, Paneth and tuft cells, which is the normal host protective mechanism against epithelial damage caused by pathogen invasions [44]. To simultaneously avoid over-exhaustion and exert anti-inflammatory functions, *Lactobacillus* modulated the expression of AMP (defa1, reg3g, muc2) and the number of AMP-secreting cells to a more reasonable level, and this finding was confirmed by a normal intestinal structure and a typical organoid morphology.

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Interactions between commensal bacteria and the intestinal epithelium influence the epithelial response to injury through TLR signaling [45]. Among intestinal epithelial cells, enteroendocrine cells express TLR1, TLR2 and TLR4 [46], Paneth cells in the small intestinal epithelium express TLR5 [47], M cells express high level of TLR4 on the apical surface [48]. TLRs are the first PRRs to detect the presence of *Salmonella [49, 50]*. Moreover, *Salmonella* has conformed to exploit TLR2 and TLR4 and manipulate the downstream transcription factor Myd88 to induce intestinal inflammation and AMP secretion [51]. Previous study indicated that *L. amylovorus* blocked ETEC-induced increase in IL-1β by inhibiting TLR4 signaling [52]. We therefore speculated whether *L. acidophilus* ATCC4356 defends against *Salmonella* through TLR2 and TLR4 to maintain intestinal homeostasis. Consistent with this hypothesis, our study detected high expression levels of TLR2, TLR4 and MyD88 in mice challenged with *Salmonella*. Compared with *Salmonella* challenge alone, pretreatment with *L. acidophilus* decreased TLR2 and TLR4 expression during *Salmonella* infection. The phenomenon is consistent with the notion that TLR4 downregulation is important for the resolution of inflammation and the repair of membrane damage. These results implied that *L. acidophilus* impaired the mechanism of *Salmonella* infection, which is initiated with intestinal inflammation and TLR activation and results in severe intestinal damage [53].

The Wnt/β-catenin pathway plays a key role in maintenance of the intestinal stem cell phenotype and differentiation [54]. Moreover, Defa1 secretion by Paneth cells is a direct target of the Wnt pathway. Appropriate Wnt activity is necessary for orderly differentiation, and deviations in activity leads to impaired differentiation [55]. A previous study indicated

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that *S. typhimurium* infection increased the expression of proliferation marker PCNA and upregulated the expression of β-catenin [56-58]. Moreover, overexpression of Wnt agonists causes hyperplasia of precursor cells (transit amplifying cell), and this effect might be attributed to AvrA [58, 59]. *S. typhimurium* SL1344 containing AvrA increased the number of $PCNA^{+}$ TA cells, which is consistent with this study's observation that the Wnt pathway was overactivated by an increase in the expression of β-catenin. We further found that *L. acidophilus* ATCC4356 alleviated hyperactivation of the Wnt/β-catenin pathway to restrain the excessive proliferation and thus maintain homeostasis and appropriate proliferation.

However, the mechanism by which *Salmonellae* induced the over activation of Wnt signal and lactobacillus relieved it remind unclear. As a gram-negative bacteria, *Salmonella* may infect the intestinal epithelial through the activation of TLR4 [60], which was verified in our mice experiment. We found that *Salmonella* infection increased the TLR4 expression, as well as TNF- alpha both in intestinal organoids and mice small intestine. The results were also verified through treatment with TLR4 inhibitor atractylenolide I. The increased expression of Wnt3 and loss of Lgr5 after infection with *Salmonella* were all reversed with atractylenolide I. These results indicated that *Salmonella* may activate the Wnt signal pathway through TLR4. The protective and modulatory effect of *Lactobacillus* on intestinal barrier were also confirmed with the application of TLR2 inhibitor CU-CUPT22 (Fig. 5E and 5F). We guess that these bacteria cause the activation of Wnt signal by indirect effect with TLRs.

In order to further verify that *S. typhimurium* causes damage by increasing the level of TNF-α, TNF-α was added to the supernatant of intestinal organoids. The morphological

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changes showed that the damage effect of TNF-α was similar to that of *S. typhimurium* infection. TNF-α treatment could also promote cell apoptosis, while *Lactobacillus* alleviated this phenomenon. DAPT and Dll4 respectively promoted or inhibited the production of secretory cells to enhance and weaken the effect of *Lactobacillus* with adjusting the expression of Muc2, Defa1 and reg3g respectively. This result confirmed the protectory effect of *L. acidophilus* ATCC4356 on intestinal epithelial. In summary, we demonstrated that *Salmonella* exploits TLR4 to trigger replication and inflammation in the murine intestine due to enhancement of TNF-α secretion and crypt hyperactivation through the Wnt/β-catenin pathway. *L. acidophilus* ATCC4356 modulated the Wnt/β-catenin pathway and affected the proliferation of the intestinal epithelium and the differentiation of Paneth and goblet cells to alleviate intestinal inflammation and crypt hyperplasia induced by *S. typhimurium* infection. The results of this study provide insights indicating that a clinical treatment that include *L. acidophilus* in addition to antibiotics might be effective for curing diarrhea and improve intestinal inflammation [61].

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Author Contributions

Xiaoxi Lu, Shuang Xie: take part in the research design, conducted research and analyzed data. Lulu Ye: cell isolation and culture. Linda Zhu: ELISA test and RT-PCR. Qinghua Yu: designed research, conducted research, wrote the paper and had primary responsibility for final content.

Conflict of interest

The authors declare no conflict of interest.

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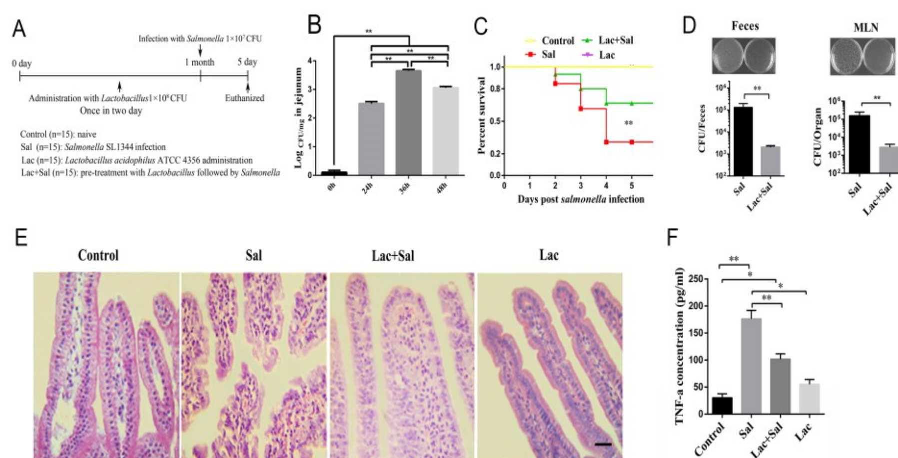

Fig. 1 ***Lactobacillus*** **provided protection against** ***S. typhimurium*** **infection in mice.** (A) The flow diagram of experimental procedure. The mice were administrated with PBS (control, Con), infected with *S. typhimurium* SL1344 (10^7 CFU) (Sal), or *L. acidophilus* ATCC4356 (10^8 CFU) (Lac) respectively or per-treated with *Lactobacillus* followed by *Salmonella* (Lac+ Sal), n=15 per group. (B) *L. acidophilus* ATCC4356 (10^8 CFU) were orally administrated to mice once and then the colonization ability of *L. acidophilus* ATCC4356 was detected in jejunum. (C) Time course of survival rate of mice. (D) *Salmonella* burden in the feces and MLN were determined on streptomycin agar plates post infection at 5 days. The result shows the CFU per gram fecal sample (scale bar represents 100 μm). Graph on the right showed quantification of the above staining. (E) HE staining showing histology of mice jejunum, scale bar represents 100 μm. (F) TNF-α detection via ELISA in mice intestinal contents. Data are mean ± SD; *$P < 0.05$, **$P < 0.01$. Data combined from at least three independent experiments unless otherwise stated.

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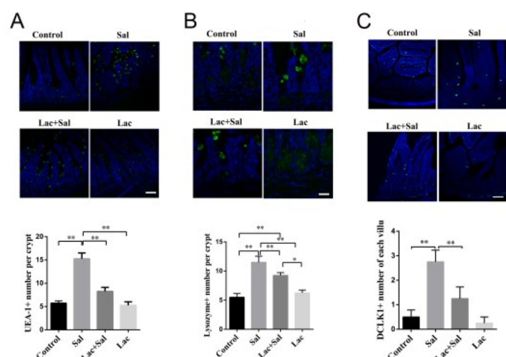

Fig. 2 ***Lactobacillus*** **alleviated secretory cells hyperplasia respond to the** ***S. typhimurium*** **infection** ***in vivo.*** Jejunum cross-sections from mice were stained with UEA-1 for goblet cells (A), lysozyme for Paneth cells (B) and DCLK1 for tuft cell (C). Data are mean ± SD.; *P < 0.05, **P < 0.01. Data combined from at least three independent experiments unless otherwise stated.

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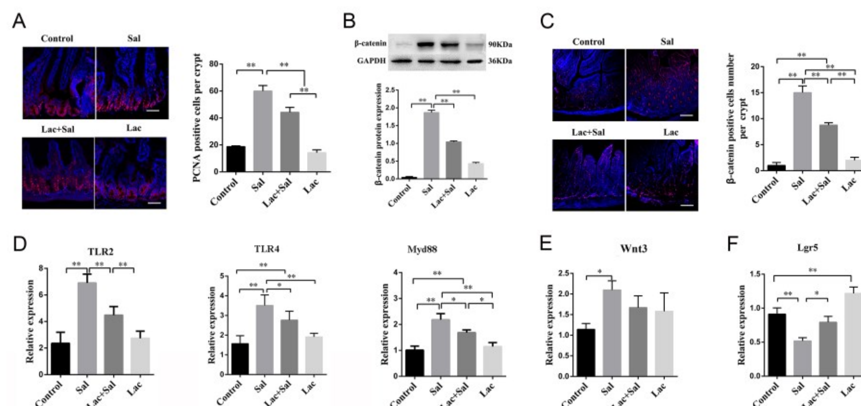

Fig. 3 The regulation effect of *L. acidophilus* on Wnt/β-catenin pathway and intestinal proliferation in mice. (A) Left: Cross-sectional view of mice intestinal crypt bottoms with co-immunofluorescence (co-IF) showing PCNA staining, Scale bar: 100 μm. Right: Graph shows quantification of the PCNA staining and (B) Western blot analysis of β-catenin expression in mice intestinal mucosa. The housekeeping gene GAPDH was used as loading control. (C) Jejunum cross-sections from mice were stained with β-catenin for detecting Wnt signaling. (D) mRNA expressions of TLR2, TLR4 and MyD88 analyzed by RT-qPCR in mice. (E) The Wnt3 mRNA expression was tested by RT-qPCR. (F) The Lgr5 mRNA expression was tested by RT-qPCR. Data are mean ± SD; *P < 0.05, **P < 0.01. Data combined from at least three independent experiments unless otherwise stated.

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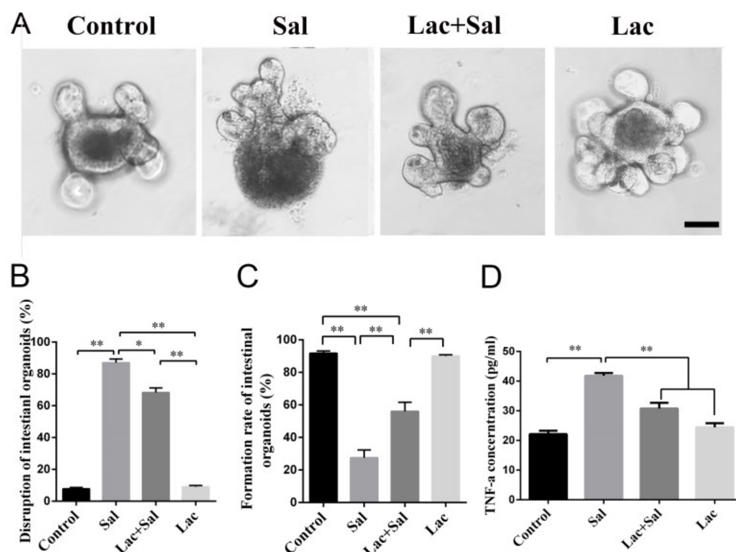

Fig. 4 *Lactobacillus* ameliorated disruption of intestinal organoids infected with *Salmonella.* The intestinal organoids were pre-treated *L. acidophilus* ATCC 4356 for 12 h before SL1344 infection. (A) Morphology of organoids was observed after *Salmonella* infection at 24 h, n = 10 wells per group. (B, C) Statistical analysis of organoids. Scale bar: 50 μm. (D) The concentration of TNF-α was measured via ELISA in cell supernatant. Data are mean ± SD; *$P < 0.05$, **$P < 0.01$. Data combined from at least three independent experiments unless otherwise stated.

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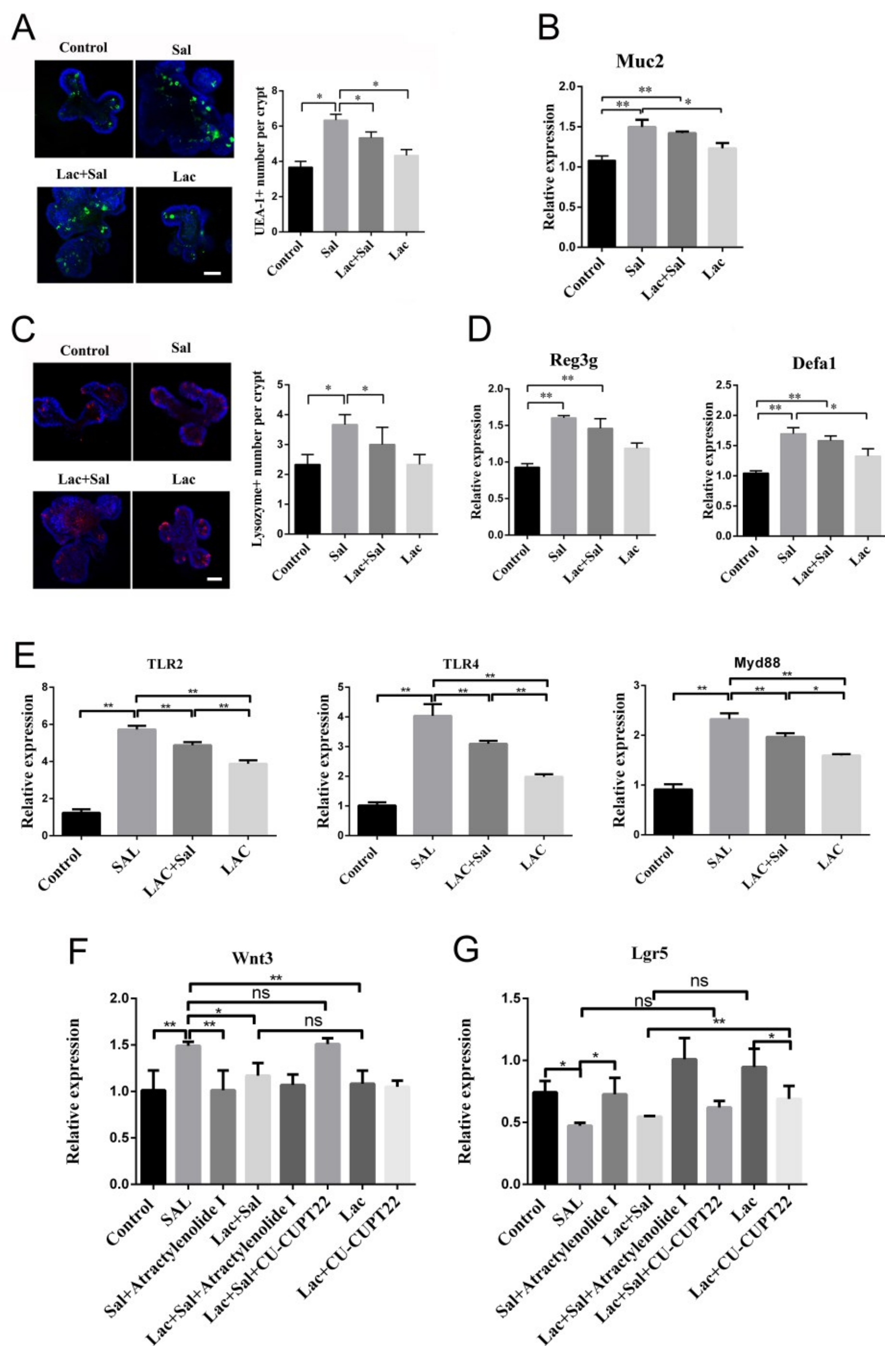

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Fig. 5 The effect of *Lactobacillus* on antimicrobial peptides expression *in vitro.*

Immuno-histological staining for goblet cells with marker UEA-1 (A) in organoids (scale bars represent 50 μm). Graph shows quantification of fluorescence positive cells. n = 10 wells per group. RT-qPCR analysis of Muc2 (B). Immuno-histological staining for Paneth cells with marker lysozyme (C) in organoids (scale bars represent 50 μm). Graph shows quantification of fluorescence positive cells. n = 10 wells per group. (D) RT-qPCR analysis of antimicrobial peptides (Reg3g and Defa1). (E) mRNA expressions of TLR2, TLR4 and MyD88 analyzed by RT-qPCR in intestinal organoids. RT-qPCR analysis of Wnt3 (F) and Lgr5 (G) with TLR2 inhibitor (CU-CUPT2) and TLR4 inhibitor (atractylenolide I). Data are mean ± SD; $*P < 0.05$, $**P < 0.01$. Data combined from at least three independent experiments unless otherwise stated.

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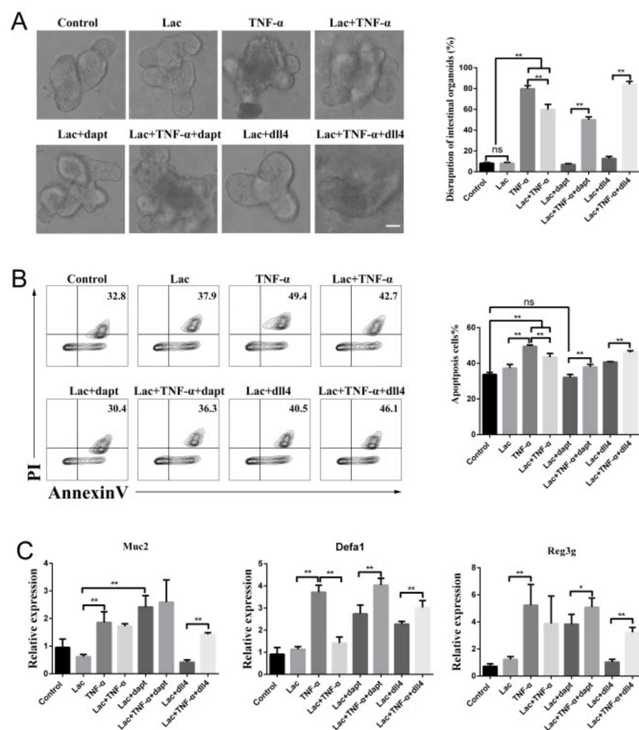

Fig.6 *Lactobacillus* protected disruption of intestinal organoids against TNF-α damage. The intestinal organoids were pre-treated *L. acidophilus* ATCC 4356 for 12 h before TNF-α damage. (A) Morphology of organoids was observed after TNF-α treatment, n = 10 wells per group. Statistical analysis of organoids. Scale bar: 50 μm. (B) Apoptosis of organoids was detected and analyzed after TNF-α treatment, n = 10 wells per group. (C) The Muc2, Defa1 and Reg3g mRNA expression was tested by RT-qPCR. Data are mean ± SD; $*P < 0.05$, $**P < 0.01$. Data combined from at least three independent experiments unless otherwise stated.

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Table 1 Primer sequences used for RT-qPCR

Target genes	Primer sense (5'-3')	Primer antisense (5'-3')
Defa1	TCAAGAGGCTGCAAAGGAAGAGAAC	TGGTCTCCATGTTCAGCGACAG
Muc2	ACGATGCCTACACCAAGGTC	TGATCTTTACATGTTCCCA
Reg3g	ATGCTTCCCCGTATAACCATCA	GGCCATATCTGCATCATACCAG
L. acidophilus ATCC4356	CTTCGGTGATGACGTTGGGA	CCAATGTGGCCGATCAGTCT
TLR4	TTCAGAGCCGTTGGTGTATC	CCCATTCCAGGTAGGTGTTT
TLR2	CAGCTTAAAGGGCGGGTCAGAG	TGGAGACGCCAGCTCTGGCTCA
MyD88	CCTGCGGTTCATCACTAT	GGCTCCGCATCAGTCT
Wnt3	CTCGCTGGCTACCCAATTTG	CTTCACACCTTCTGCTACGCT
Lgr5	CCTACTCGAAGACTTACCCAGT	GCATTGGGGTGAATGATAGCA
GAPDH	ATGGTGAAGGTCGGTGTGAA	TGGAAGATGGTGATGGGCTT

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