Lactobacillus rhamnosus GR-1 attenuates foodborne Bacillus cereus-induced the NLRP3 inflammasome activity in bovine mammary epithelial cells by protecting intercellular tight junctions

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Research Article

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Abstract

Background: *Bacillus cereus* (*B. cereus*) is one of the important pathogens of human food poisoning that causes diarrhea and vomiting. *B. cereus* can induce mastitis with strong survival ability, which cannot be inactivated by high-temperature short-time pasteurization conditions. Therefore, *B. cereus* has the opportunity to enter the market through pasteurized milk and dairy products, causing great hidden dangers to food safety and human health.

Results: In this study, *B. cereus* 2101 (BC) was isolated from dairy cow milk samples with mastitis. BC secreted HBL, NHE, InhA, Hemolysin A, Hemolysin III, CytK, EntFM, and tolerated to AMP, AMC, AZI, CZ, CIP, STR, and TE. BC grew rapidly with strong hemolysis, which caused great difficulties in the prevention of mastitis and food security. MAC-T cells were treated with BC and / or *Lactobacillus rhamnosus* GR-1 (LGR-1), and the results showed that pretreatment with LGR-1 protected the integrity of tight junction and the expression of ZO-1 and Occludin destroyed by BC. Besides, we also found out that pretreatment with LGR-1 reduced the expression of NLRP3, ASC, Caspase1 p20, GSDMD p30, inflammatory factors (IL-1 β and IL-18) and cell death induced by BC. These indicators are related to the NLRP3 inflammasome activity. Moreover, pretreatment with LGR-1 reduced the NLRP3 inflammasome activity, and increased the expression of ZO-1 and Occludin induced by LPS + ATP stimulation. MAC-T cells were transfected with NLRP3 siRNA for 5 h or MCC950 (NLRP3 inhibitor) for 0.5 h and / or treated with BC and / or LGR-1 for 3 h. NLRP3-siRNA transfection and MCC950 attenuated the NLRP3 inflammasome activity induced by BC. The expression of inflammatory cytokines and cell death suggested that the inflammatory pathway might play an important role in the induction of NLRP3 inflammasome by BC and the protection of LGR-1.

Conclusions: These results suggest that LGR-1 might be a probiotic for the alternative to antibiotics and could be administered to prevent mastitis in dairy cows to ensure food security.

Introduction

Milk plays an important role in human diets by providing energy, protein, and other key nutrients [1]. However, the spores of *Bacillus cereus* (*B. cereus*) in raw milk may not be terminated by pasteurization at high temperature for a short time (72°C for 15 s), they can germinate and may grow to high level in pasteurized milk and refrigerated dairy products, which causes potential safety hazard in milk consumption [2].

B. cereus is a Gram-positive rod-shaped bacterium, which could survive in harsh environmental conditions by producing endophytes and forming biofilms [3–5]. At the same time, it is an important cause of food poisoning in humans [6]. *B. cereus* is an important reason of 1.4%-12% foodborne diseases worldwide [7; 8]. Food poisoning caused by *B. cereus* could be manifested as emetic or diarrheal syndrome, it seriously affects human health [8]. Numerous studies have shown that *B. cereus* may cause mastitis in dairy cows [9; 10].

B. cereus produces a collection of virulence factors, including haemolysin BL (HBL), non-haemolytic enterotoxin (NHE), enterotoxin FM, hemolysin A, hemolysin III, cytotoxin K, and others [6; 7]. HBL and NHE can assemble on the cell membrane of the host, induce pore formation, and facilitate potassium (K^+) efflux, which subsequently activates the immune sensor NOD-like receptor family member pyrin domain-containing protein 3 (NLRP3) [6; 11; 12]. NLRP3 recruits apoptosis-associated speck-like proteins, which contain caspase recruitment and activation domain (ASC) and Caspase-1 to form inflammasome complexes [6; 13–15]. The activation of NLRP3 inflammasome leads to the autoproteolysis of Caspase-1, which cuts interleukin (IL) -1β , IL-18, and pro-pyroptotic factor gasdermin D (GSDMD) into their active forms [14; 16]. The active N-terminal fragment of GSDMD forms pores on the cell membrane, leading to a cell death called pyroptosis [6; 17]. The K^+ efflux caused by pore-forming toxins produced by bacteria and these inflammatory caspases oligomerization caused by lipopolysaccharides (LPS) produced by cell walls of Gram-negative bacteria could lead to the activation of NLRP3 inflammasome [18]. NLRP3 inflammasome complexes could be activated by a series of pathogen-associated molecular patterns (PAMPs) [11; 18].

As the first line of defense for breast self-protection, bovine mammary epithelial cells are critical in the initial stage of pathogen infection [19]. Tight junctions are intercellular junctions between epithelial cells at the top region of cell-cell contacts, which could construct the epithelial barrier and maintaining epithelial polarity to protect epithelial cells [20]. Its main role is to form permeability barrier, so as to determine the selective permeability of epithelial cells [21]. The tight junctions transmembrane protein Occludin and the tight junctions protein Zonula occludens-1 (ZO-1) play an indispensable role in constituting the cytoskeleton [22]. In previous studies of probiotics, it was found that they could regulate epithelial barrier function by stimulating the tight junctions between epithelial cells, thereby maintaining the integrity of epithelial cells [23; 24].

Lactobacillus rhamnosus GR-1 (LGR-1) is the most studied female vaginal probiotics, which could inhibit the growth and prevent the adhesion of various bacterial pathogens [24]. *Lactobacilli* could protect the integrity of tight junctions by wrapping on the cell surface to prevent the damage of pathogens [25]. Studies have shown that lactic acid bacteria could upregulate ZO-1 and Occludin [26; 27]. At present, most studies on LGR-1 focus on regulating the inflammatory pathway, while little attention is paid to its protective effect on tight junctions. Studies have shown that LGR-1 could reduce *E. coli*-induced Caspase-1 activation and IL- 1β and IL-18 production, and inhibit *E. coli*-induced pyroptosis [28; 29]. Moreover, some studies indicate that LGR-1 could ameliorate *E. coli*-induced cell ultrastructure damage, reduce the percentage of primary bovine endometrial epithelial cells apoptosis, inhibit the inflammatory response, and then effectively alleviate the activation of NLRP3 inflammasome and apoptosis [30; 31]. Additional studies have shown that LGR-1 inhibits biofilm formation on *Candida albicans* strains on abiotic surfaces and *Salmonella enterica* serovar *Typhimurium* [32; 33]. Therefore, the aim of this study was to investigate the potential molecular mechanism of LGR-1 attenuated the *B. cereus*-induced the NLRP3 inflammasome activity of bovine mammary epithelial cells by protecting intercellular tight junction.

Materials And Methods

MAC-T culture

The bovine mammary epithelial cell line (MAC-T) cells were incubated in DMEM/Ham's F-12 medium (GE Healthcare Life Sciences HyClone Laboratories, Utah, USA) supplemented with 10% fetal bovine serum (ThermoFisher Scientific, Rockford, USA), 100 U/mL of penicillin and streptomycin (Invitrogen, Carlsbad, CA, United States) at 37°C in an atmosphere of 5% CO₂.

Isolation and culture of *B. cereus*

B. cereus 2101 (BC) was isolated from milk samples of dairy cows with mastitis from youran dairy Co., Ltd. (Daqing, China). Subsequently, the strain was sent to the Beijing Tianyi Huiyuan Biotechnology Co., Ltd (Beijing, China) for Bacterial 16S rRNA sequencing. The sequence was subjected to Nucleotide BLAST alignment on NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to obtain the strain information. BC was grown in Luria–Bertani (LB) broth (AOBOX, Beijing, China) overnight under aerobic conditions at 37°C and 200 rpm shaking. It was used to stimulate MAC-T for 3 h with multiplicity of infection (MOI) of 5.

Lactobacillus rhamnosus GR-1 culture

ATCC 55826 LGR-1 was purchased from the American Center for the Preservation of Typical Cultures (Manassas, V A, United States), and grown at 37°C for 24 h in de Man, Rogosa and Sharpe (MRS) broth (AOBOX, Beijing, China) under microaerophilic conditions. After overnight incubation, LGR-1 was diluted into 1:100 with MRS broth and subcultured for about 12 h until mid-logarithmic (optical density at 600 nm (OD₆₀₀) = 0.5). It was used to pretreated with MAC-T cells for 3 h with MOI of 100.

Biosecurity statement

Clinical isolates of *Bacillus cereus* were treated strictly in accordance with the State Council of the People's Republic of China, Regulations on biological safety of Pathogen Microbiology laboratory (000014349/2004 – 00195). Conduct all necessary safe operations on it to avoid pathogen transmission and infection

LDH assay

To examine cell death under different treatments, LDH levels were measured using LDH Cytotoxicity Assay Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Determination of growth curves

Sterilized turbidity tubes were added 1 mL LB broth and BC solution. The turbidity was adjusted to 0.2 McFarland Standard (MCF) by turbidity meter (BioMérieux, Durham, NC, United States). 198 µL of LB broth was added to each well into a 96-well plate and then 2 µL of BC solution with a turbidity of 0.2 was added to each well. The experiment was repeated three times. The plate was placed in the microplate reader (Tecan, Switzerland) and measured OD₆₀₀ every hour for a continuously 31 h measurement.

Strain characteristics

BC was cultured on LB nutrient agar (AOBOX, Beijing, China) supplemented with 5% sheep blood (Solarbio, Beijing, China), and incubated upside down overnight at 37°C. BC was differential staining according to the instructions of Gram Staining Kit (Solarbio, Beijing, China).

Virulence Factors of *B. cereus* 2101

The DNA of BC was extracted with TIANamp Bacterial DNA Kit (TIANGEN, Beijing, China). The virulence gene primers (Table 1) was used for PCR virulence gene detection [34; 35]. PCR products were analyzed by 1% agarose gel electrophoresis for 30 min at 125 V in 1× Tris–acetate–EDTA buffer, stained with ethidium bromide and photographed under UV transillumination.

Table 1
Primer sequences of the virulence genes

Gene name	Direction	Primer sequences (5'-3')	Length (bp)
HBLA	F	CGGGATCCGCAGTCATACCAATAGAACTTTTGC	1351
	R	CCCTCGAGTCAGTTCATTATATTTTGTACTTTGTCTTTATACAC	
HBLB	F	CGGAATTCTCACCAGTAACAACCTTTGCAAGTGAA	1072
	R	CCCTCGAGCTATTTTTGTGGAGTAACAGTTTCCACTTTT	
HBLD	F	CGGGATCCGCATTTGCACAAGAAACGACCG	1156
	R	CCCTCGAGCTACTCCTGTTTAAAAGCAATATCTTTTGAAATGAA	
NHEA	F	CGGGATCCACGAGTTGCTTCATTCTGTAAAGC	1132
	R	CCCTCGAGTTAATGTACTTCAACGTTTGTAACGTAATCTTCAAAT	
NHEB	F	CGGAATTCAATATTATGCCGGCTCATACGTATGCA	1162
	R	CCCTCGAGTTATGCTTTTTTCGTATCTACTACTTTAATATCTTC	
NHEC	F	CGGAATTCATGCCGGCTCATACGTAT	1156
	R	CCCTCGAGTTATGCTTTTTTCGTATCTACTACTTTAATATCTTCA	
lhA	F	CGGAATTCATGAGTGCTCCGTTAGCATATGCA	2344
	R	CCCTCGAGTTAACGTTTAATCCAAACAGCGCCTGC	
CytK	F	CGGAATTCCTGCTACTTACGCTCAAAC	949
	R	CCCTCGAGTTATTTTTCTCTACTAATTTCTTATTCTTCCAATCTAG	
HlyA	F	CGGAATTCGCCATTATGGCCGGACT	778
	R	CCCTCGAGTTATTCCCCTTTCCCTTTTTGTTTTAG	
Hly _{II}	F	CGGAATTCGCAATTACACATGGTATCGGTG	619
	R	CCCTCGAGTTATGCTGTAGGTAAGACATAAAAGAGTACA	
EntFM	F	ATGAAAAAAGTAATTTGCAGG	1269
	R	TTAGTATGCTTTTGTGTAACC	

Determination of MIC

The minimum inhibitory concentration (MIC) of BC was determined by the broth microdilution method and *Escherichia coli* ATCC 25922 was used as quality control reference. The experiment was operated strictly according to performance standards for antimicrobial susceptibility testing M100 of the clinical and laboratory standards institute (CLSI). The results of MIC for ampicillin (AMP), amoxicillin (AMC),

azithromycin (AZI), cefazolin (CZ), ciprofloxacin (CIP), gentamicin (GM), meropenem (MEM), kanamycin (KAN), streptomycin (STR) and tetracycline (TE) were examined. Three independent repetitions per trial were performed. The isolate was defined as “susceptible (S)”, “intermediate (I)” or “resistant (R)” based on MIC for each antimicrobial agent.

Drug Treatments

MAC-T cells were treated under different conditions as follows: (☐) control group without treatment (CONTROL), (☐) exposed to BC for 3 h (BC), (☐) pretreatment with LGR-1 for 3 h and exposed to BC for 3 h (LGR-1 + BC), (☐) pretreatment with LGR-1 for 3 h (LGR-1), (☐) pretreatment with MCC950 (100 nM, HY-12815, MedChemExpress, USA) for 30 min and exposed to BC for 3 h (MCC950 + BC), (☐) pretreated with MCC950 for 30 min (MCC950), (☐) pretreatment with LGR-1 for 3 h, then primed using LPS from *E. coli* 0111: B4 (500 ng/mL, L4391, Sigma-Aldrich, USA) for 4 h and stimulated with ATP (5 mM, A6419, Sigma-Aldrich, USA) for 45 min (LGR-1 + LA), (☐) primed using LPS for 4 h and stimulated with ATP for 45 min (LA), (☐) pretreatment with an increasing concentration of KCl at 5 mM, 25 mM, 50 mM and 75 mM (P9541, Sigma-Aldrich, USA) for 30 min and exposed to BC for 3 h, (☐) pretreatment with 50 mM of KCl and primed using LPS for 4 h and stimulated with ATP for 45 min. (☐) exposed to BC (MOI = 0.5, 5, or 50) for 3 h, (☐) pretreatment with LGR-1 (MOI = 1, 10, 100, or 1000) for 3 h, and then exposed to BC (MOI = 5).

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

At 3 h after BC challenge, MAC-T cells were harvested and fixed in 3% glutaraldehyde (pH = 7.4). Samples were treated following the standard SEM and TEM procedures [28].

Western Blotting

MAC-T cells were lysed in lysis buffer, composed of 1 mL of RIPA buffer, 10 μ L of phenylmethanesulfonyl fluoride (Solarbio, Beijing, China). The resulting lysates were centrifuged at 13,000 $\times$ g for 10 min at 4°C to pellet insoluble material, and the supernatants were used for Western blotting analysis. Protein concentrations were determined using a BCA Protein Assay kit (ThermoFish Scientific, Waltham, MA, USA). The following primary antibodies were used: ZO-1 (21773-1-AP, 1: 1000 dilution, Proteintech Group), Occludin (27260-1-AP, 1: 1000 dilution, Proteintech Group), NLRP3 (19771-1-AP, 1: 500 dilution, Proteintech Group), ASC (10500-1-AP, 1: 1000 dilution, Proteintech Group), Caspase-1 (sc-56036, 1: 200 dilution, Santa Cruz Biotechnology), GSDMD (20770-1-AP, 1: 2000 dilution, Proteintech Group) and mouse anti-GAPDH mAb (60004-1-Ig, 1: 5000 dilution, Proteintech Group). Horseradish peroxidase-conjugated affipure goat anti-mouse IgG (H + L) (SA00001-1, 1: 8000 dilution, Proteintech Group) or goat anti-rabbit IgG (H + L) (SA00001-2; 1: 8000 dilution, Proteintech Group) were used as secondary antibodies. Tanon 6200 chemiluminescence imaging workstation (Tanon, Shanghai, China) was used to visualize the immunoreaction bands. Densitometric values of Western blot images were obtained from 3 independent experiments using Image J software (National Institutes of Health, Bethesda, MD). Results are presented as the ratio of ZO-1, Occludin, NLRP3, ASC, GSDMD, or Caspase-1 band intensity to that of GAPDH.

Immunofluorescence

The cells were fixed with 4% paraformaldehyde, treated with 1% (v/v) Triton X-100 and then blocked with 2% BSA. MAC-T cells were then incubated with NLRP3 (1: 100 dilution), ASC (1: 100 dilution), GSDMD (1: 200 dilution), Caspase1 (1: 50 dilution), ZO-1 (1: 750 dilution) at 4°C overnight. Then the samples were incubated with secondary antibody at room temperature for 1 h. DAPI (C0065, Solarbio, Beijing, China) was used for nuclear staining. MAC-T cells were observed and photographed using a Nikon A1 confocal laser scanning microscope.

Quantitative real-time PCR

RNAiso Plus (9108, TaKaRa, Japan) was used to extract total RNA from MAC-T cells, and PrimeScript™ RT Reagent Kit (RR047A, TaKaRa, Japan) was used for RNA transcription. Next, SYBR Green PCR Master Mix (LS2062, Promega, USA) was used for real-time quantitative PCR (qPCR). The mRNA expression of IL-1β, IL-18, and NLRP3 was normalized to the mRNA expression of GAPDH. The primer sequences are shown in Table 2 and the gene expression levels were analyzed with the $2^{-\Delta\Delta CT}$.

Table 2
Primers sequences of qPCR

Gene name	Direction	Primer sequences (5'-3')
IL-1β	F	CCTCGGTTCCATGGGAGATG
	R	AGGCACTGTTCTCAGCTTC
IL-18	F	TCAGATAATGCACCCCAGACC
	R	GATGGTTACGGCCAGACCTC
NLRP3	F	ATGGTAAGTGTCCGCTGCAA
	R	ATGCCTTCTCTTCCCCGTTG
GAPDH	F	GTCTTCACTACCATGGAGAAGG
	R	TCATGGATGACCTTGGCCAG

RNA Interference

To verify the importance of NLRP3 inflammatory pathway in *B. cereus*-infected MAC-T cells, we knocked out NLRP3 by gene silencing. MAC-T cells were seeded in 6-well plates. When MAC-T cells grew to 60%-80% confluence, NLRP3-siRNA (si-NLRP3, Sense 5' - GGAGAGACCUUAUGAGAATT - 3', Antisense 5' - UUCUCAUAAAGGUCUCUCCTT - 3', GenePharma, Suzhou, China) diluted with Lipo8000™ Transfection Reagent (Beyotime Biotechnology, Shanghai, China) was transfected into MAC-T cells. After transfection for 5 h, the fresh medium was replaced and incubated at 37°C for 48 h for subsequent treatment and analysis.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.2.0. The t-test was used for comparison between two groups, and one-way ANOVA was used for three or more groups. The value is expressed as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ were considered indicative of statistical significance. All experiments were performed 3 times.

Results

Screening and characteristics of *B. cereus*

BC was isolated and identified from milk samples of dairy cows with mastitis. The results showed that BC was bar-like Gram-positive bacteria with hemolytic activity (Fig. 1A), producing HBL, NHE, InhA, Hemolysin A, Hemolysin III, CytK, and EntFM (Fig. 1B). By measuring the growth curve of BC, we found that the logarithmic phase was between 6–12 h (Fig. 1C). BC with MOI = 50 and MOI = 5 showed strong cytotoxicity in MAC-T cells (Fig. 1D). MIC indicated that BC was resistant to AMP, AMC, AZI, CZ, CIP, STR, and TE (Table 3), and had multi-antibiotic resistance. Pretreatment with LGR-1 (MOI = 100) for 3 h could effectively alleviate cell death after exposed to BC (MOI = 5) for 3 h (Fig. 1E).

Table 3
Antimicrobial susceptibility profiles of the *B. cereus* clinical isolates.¹

Antibiotics ²										
Strains	AMP	AMC	AZI	CZ	CIP	GM	MEM	KAN	STR	TE
<i>B. cereus</i> 2101	R	R	R	R	R	S	S	S	R	R
1) The isolates were defined as “susceptible (S)”, “intermediate (I)” or “resistant (R)” based on MIC for each antimicrobial agent.										
2) Ampicillin (AMP), amoxicillin (AMC), azithromycin (AZI), cefazolin (CZ), ciprofloxacin (CIP), gentamicin (GM), meropenem (MEM), kanamycin (KAN), streptomycin (STR) and tetracycline (TE).										

LGR-1 protects *B. cereus* damaged intercellular tight junctions

Tight junction is the key structure of epithelial barrier, composed of tight junction proteins, which can protect epithelial cell integrity. Through transmission electron microscopy, we found that the tight junction structures of CONTROL group, LGR + BC group, and LGR-1 group were complete, while the tight junction structure of BC group was destroyed (Fig. 2A). Immunofluorescence showed that the ZO-1 protein in CONTROL group was distributed between cells, and the structure was complete, while the integrity of ZO-1 protein in BC group was destroyed (Fig. 2B). Our WB results further support this phenomenon that compared with CONTROL group, BC group significantly reduced the protein expression of ZO-1 and Occludin, while LGR-1 + BC group significantly increased the expression of these proteins compared with BC group, indicating that pre-treatment with LGR-1 can prevent BC to destroy tight junction proteins (Fig. 2C-E; $P < 0.01$).

LGR-1 attenuated *B. cereus*-induced inflammatory responses

Transmission electron microscopy further exhibited membranolysis, loss of cytoplasmic content, chromosome condensation, and chromatin margination in MAC-T cells stimulated with BC (Fig. 2A). Western blotting analysis showed that the protein expression of NLRP3, ASC, Caspase1 p20, and GSDMD p30 was increased in BC group compared with CONTROL group, indicating that NLRP3 inflammasome activity was induced by BC stimulation (Fig. 3A and B; $P < 0.05$). The expression of these proteins in LGR-1 + BC group significantly reduced compared with BC group, and pre-treated of LGR-1 did not affect the NLRP3 inflammatory pathway (Fig. 3A and B). Further, we used immunofluorescence to explore the effect of LGR-1 on the NLRP3 inflammasome activity induced by BC. We found that BC formed the ASC speck, which was a hallmark of NLRP3 inflammasome activity (Fig. 3E) [36]. The expression levels of NLRP3, GSDMD, and Caspase1 were increased in BC group, and were reduced in LGR-1 + BC group (Fig. 3D, F, and G). Similarly, the expression of the NLRP3 pathway target genes IL-1 β and IL-18 was also increased in BC group, while LGR-1 attenuated the *B. cereus*-induced expression of IL-1 β and IL-18 (Fig. 3C; $P < 0.01$).

B. cereus-induced NLRP3 inflammasome activity requires K⁺ efflux

Studies have shown that the efflux of K⁺ is considered to be the central mechanism for the activation of NLRP3 inflammasome [18; 37]. We observed that the addition of extracellular K⁺ inhibited the NLRP3 inflammasome activity induced by BC in a dose-dependent manner, and effectively inhibited the expression of IL-1 β and IL-18 and cell death at 50 mM K⁺ (Fig. 4A-C; $P < 0.05$). The appropriate addition of extracellular K⁺ also inhibited the NLRP3 inflammasome activity by LPS + ATP, which was consistent with the above results. LGR-1 protected the integrity of the tight junction structure and ensured the normal cell selective permeability.

LGR-1 inhibits inflammatory responses by protecting tight junctions

LGR-1 attenuated *B. cereus*-induced NLRP3 inflammasome activity. Therefore, we further explored the effect of LGR-1 on NLRP3 inflammasome activity. We found that pre-treatment with LGR-1 did not affect the NLRP3 inflammatory pathway, but significantly reduced NLRP3 inflammasome activity and expression of IL-1 β and IL-18 caused by LPS + ATP, indicating that LGR-1 did not inhibit the activation of inflammatory cells, but protected cells in other ways (Fig. 5A-G; $P < 0.05$). Therefore, we explored the tight junction proteins and found that when the NLRP3 pathway was stimulated with LPS + ATP, pre-treatment with LGR-1 could significantly increase the protein contents of ZO-1 and Occludin (Fig. 2F-H; $P < 0.05$).

***B. cereus*-induced inflammatory responses by through NLRP3**

BC stimulation increased the protein expression of NLRP3, Caspase1 p20, and GSDMD p30 (Fig. 6A and D-F; $p < 0.05$). Compared with BC group, NLRP3 and Caspase1 p20 in MCC950 + BC group decreased. Similarly, the mRNA expression of IL-1 β and IL-18 increased in BC group, but decreased in MCC950 + BC group (Fig. 6B and C; $p < 0.01$). Compared with the si-control group, transfection with NLRP3 siRNA reduced the NLRP3 expression of mRNA and protein (Fig. 7A-C; $p < 0.01$). Compared with CONTROL group, the protein expression of Caspase1 p20 in BC group was increased, while in si-NLRP3 + BC group was significantly decreased (Fig. 7D and H; $P < 0.01$). Furthermore, compared with CONTROL group, the mRNA abundance of IL-1 β and IL-18 in BC group was significantly increased, whereas in si-NLRP3 + BC group was significantly decreased (Fig. 7E and F; $P < 0.001$). Compared with BC group, cell death in si-NLRP3 + BC group was significantly decreased, which is consistent with the above changes in Caspase1 and inflammatory cytokine abundance (Fig. 7G; $P < 0.001$).

Discussion

B. cereus is often found in raw milk, and pasteurization cannot achieve complete sterilization, so the existence of *B. cereus* affects the shelf life of pasteurized milk [38]. As a food-borne pathogen, *B. cereus* can cause diarrheal and emetic syndromes through food entering the intestine, which has adverse effects on human health [7]. The main cause of the disease is that *B. cereus* can produce a variety of virulence factors to play a pathogenic role. Some studies have found that *B. cereus* could produce pore-forming toxins, induce the potassium efflux, and activate NLRP3 inflammasomes by forming pores, resulting in pyroptosis [11; 12]. When *B. cereus* infects the intestine, the intestinal microenvironment promotes the production of toxins, thereby causing damage to epithelial cells, lysing intestinal epithelial cells, and leading to diarrhea [39–41]. BC used in the experiment can secrete HBL, NHE, InhA, Hemolysin A, Hemolysin III, CytK, and EntFM, and has the characteristics of multi-drug resistance, rapid growth, and strong hemolysis, which has brought great difficulties to elimination and prevention.

LGR-1 is currently the most studied female vaginal probiotics [24]. Previous studies have found that LGR-1 inhibits pathogen growth by adhering to mucosal surfaces [24; 42]. The integrity of the epithelial lining is very important in our health protection. When it is disrupted, pathogens enter tissues and bloodstream, leading to inflammation and disease [25]. Tight junction is a key part of constructing epithelial barrier, which forms permeability barrier and determines the selective permeability of epithelial cells [19]. Li *et al.* found that *Lactobacillus rhamnosus* SHA113 restored damaged intestinal barrier and the changes of the epithelial cell cytoskeleton caused by *Escherichia coli* QBQ009, and also regulated the expression of tight junction proteins ZO-1 and Occludin [43]. Similarly, after pre-treatment with *Lactobacillus plantarum*, it was found that ZO-1 and Occludin significantly increased around the tight junction structure, forming the paracellular seal between epithelial cells [44]. There were also studies showing that *Lactobacillus plantarum* prevented EIEC-induced protein expression and rearrangement of Occludin and ZO-1 [45]. In

this study, we found that BC could destroy tight junction and significantly down-regulate the expression of ZO-1 and Occludin, while pre-treatment with LGR-1 could offset this damage, indicating that LGR-1 could effectively protect tight junctions and maintain epithelial cell barrier.

NLRP3 is unique in the NLR receptor family because it is the member indirectly activated by numerous pathogenic and sterile inflammatory signals, including PAMPs of bacteria and viruses, pore-forming toxins, extracellular ATP, K⁺ efflux, and other signals [46]. Inflammasomes are important platforms for inflammatory signal transduction. When they detect body or tissue damage, they release injury mediators to activate inflammatory response [14]. NLRP3 inflammasome recruits contain caspase recruitment and activation domains ASC and Caspase-1, forming an inflammasome corpuscle complex [36]. The activation of NLRP3 leads to the cleavage of Caspase-1, the release of IL-1 β and IL-18, and the cleavage of GSDMD and its transformation into an active form. Then, the active N-terminal fragment of GSDMD forms pores on the cell membrane, resulting in pyroptosis [6]. Studies have shown that *B. cereus* infected cells are related to NLRP3 inflammasome activity [11; 12]. The expression levels of NLRP3, ASC, the cleavages of Caspase-1 and GSDMD, IL-1 β , and IL-18 were increased significantly after the stimulation with BC to MAC-T cells, indicating that BC activated NLRP3 inflammasome and caused pyroptosis. Similarly, Zhao *et al.* found that the virulent *B. cereus* strain from deep-sea cold seep could lead to the activation of NLRP3 inflammasome *in vivo* and *in vitro* [47].

There is abundant evidence showing that, LGR-1 can effectively inhibit NLRP3 inflammasome activity. Li *et al.* found that LGR-1 pretreatment eliminated the activation of NLRP3 inflammasome induced by *Escherichia coli* [31]. Similarly, Wu *et al.* found that preincubation with LGR-1 effectively inhibited NLRP3 inflammasome and Caspase-1 activation caused by *E. coli* O111 infection in primary bovine mammary epithelial cells [28; 29]. We found that pre-treatment with LGR-1 could effectively alleviate the increase of NLRP3, ASC, the cleavages of Caspase-1 and GSDMD, IL-1 β , and IL-18 caused by BC, while pre-treatment with LGR-1 alone did not have an effect. LGR-1 could protect the tight junction structure and increase the expression of ZO-1 and Occludin, but it didn't affect the activation of NLRP3 inflammasome, indicating that it reduced the stimulation of BC by protecting tight junctions, thereby weakening the activation of NLRP3 inflammasomes and reducing pyroptosis. Previous studies revealed that LPS can destroy the integrity of endothelial barrier, damage the tight junction, reduce the expression of ZO-1 and Occludin, and change the permeability of cell barrier [48–50]. In this study, LPS + ATP was used to stimulate the NLRP3 inflammasome activity. Pretreatment with LGR-1 could reduce the activation of NLRP3 inflammation and increase the expression of ZO-1 and Occludin, indicating that LGR-1 protects the tight junction, thereby reducing the activation of NLRP3 inflammasome.

Epithelial cells establish selectively permeable barriers to support cell nutrient absorption and waste secretion [51]. The selective permeable barrier is realized by the intercellular tight junction structure, which regulates the permeability of cells. When it is destroyed, the permeation of toxins leads to inflammation [52]. Mathur *et al.* and Fox *et al.* found that the activation of NLRP3 inflammasome induced by virulence factors HBL and NHE of *B. cereus* required K⁺ efflux [11; 12]. Studies have found that NHE induces pore formation, forming conductive openings and penetrating cations [53; 54]. Furthermore, we supplemented

extracellular K^+ to inhibit the K^+ efflux and then stimulated by BC. We found that IL-1 β , IL-18, and cell death decreased with the increase of K^+ dose. Similarly, K^+ also inhibited IL-1 β , IL-18, and cell death after the stimulation of LPS + ATP. We speculated that LGR-1 prevented the K^+ efflux caused by virulence factors of BC by protecting tight junctions, which may also be one of the ways to prevent the activation of NLRP3 inflammasome.

In order to prove that the NLRP3 inflammasome is activated after MAC-T cells infected by BC. In this study, pre-treatment with MCC950 (NLRP3 inhibitor) and knockdown of NLRP3 by si-NLRP3 were used to inhibit the expression of NLRP3, thereby directly inhibiting the activation of NLRP3 inflammasome. We found that BC caused pyroptosis by activating NLRP3 inflammasome, which as evidence to the decrease of Caspase1 p20 and GSDMD p30 after inhibition of NLRP3 by MCC950. Subsequently, we further used si-NLRP3 to silence NLRP3, and found that the expression of inflammatory cytokines and cell death were significantly reduced after silencing. However, there was no significant difference in the expression of inflammatory cytokines and cell death between the LGR-1 group and the BC group, indicating that LGR-1 did not affect MAC-T cells when NLRP3 was silenced. Therefore, these results confirmed that BC could activate NLRP3 inflammasome to induce cell pyroptosis and LGR-1 could inhibit the activation of NLRP3 inflammasome induced by BC.

Conclusion

In summary, this experiment provides important evidence for LGR-1 to reduce the activation of NLRP3 inflammasomes by protecting intercellular tight junctions induced by BC. Mechanisms of action included protecting intercellular tight junction, preventing K^+ efflux, and inhibiting the activation of NLRP3 inflammasome and pyroptosis (Fig. 8). The cytoprotective effect of LGR-1 at least partly depended on the protection of tight junctions. In this study, BC was isolated from milk samples of dairy cows with mastitis caused obvious harms to MAC-T cells, and probiotics LGR-1 has supplied prominently prevention, which provided theoretical basis for the prevention of mastitis and food safety.

Abbreviations

B. cereus; *Bacillus cereus*; NLRP3; NOD-like receptor family member pyrin domain-containing protein ; ASC; Apoptosis-associated speck-like protein, which contain caspase recruitment and activation domain; IL; Interleukin; GSDMD; Gasdermin D; LPS; Lipopolysaccharides; ATP; Adenosine 5'-triphosphate; ZO; Zonula occludens; LGR-1; *Lactobacillus rhamnosus* GR-1; MOI; Multiplicity of infection; MRS; De Man, Rogosa and Sharpe; MIC; Minimum inhibitory concentration; siRNA; Short interfering RNA; SEM; Scanning electron microscopy; TEM; Transmission electron microscopy; qPCR; Real-time quantitative PCR.

Declarations

Ethical Approval and Consent to participate

This work has received approval for research ethics from Ethical Committee of the College of Veterinary Medicine, China Agricultural University (CAU), Beijing and the study was conducted according to standard ethical guidelines implemented at CAU (SYXK, 2016-0008).

Consent for publication

Not applicable.

Availability of supporting data

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

QS, NL, and JFW conceived the study. QS and NL collected the samples. QS, XW, and YHZ analyzed the data. QS, NL, and XW conducted the experiment. QS, NL, and JFW wrote and prepared the manuscript. The authors read and approved the final manuscript.

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Figures

Figure 1

Lactobacillus rhamnosus GR-1 (LGR-1) alleviated *Bacillus cereus* 2101 (BC)-induced cell death. The MAC-T cells were treated with BC (MOI = 0.5, 5 or 50) and/or LGR-1 (MOI = 1, 10, 100 or 1000) for 3 h. (A) The blood plate showed that the strain was hemolytic. The strain was rod-shaped observed by scanning electron microscopy and transmission electron microscopy; scale bar in the lower left corner. The color was purple after staining with Gram Staining Kit. (B) The virulence factors of BC. M. Marker; 1. HBLA; 2. HBLB; 3. HBLD; 4. NHEA; 5. NHEB; 6. NHEC; 7. InhA; 8. CytK; 9. HlyA; 10. Hly ϕ ; 11. EntFM. (C) Growth curves of BC. (D) Cell death induced by BC. (E) Cell death induced by LGR-1 and/or BC. One-way ANOVA was used for comparison between groups. The data were mean $\pm$ SEM of three independent experiments. * * * $P < 0.001$.

Figure 2

LGR-1 protects BC damaged intercellular tight junctions. The MAC-T cells were treated with BC (MOI = 5) or LPS (500 ng/mL) + ATP (5 mM) and/or LGR-1 (MOI = 100). **(A)** The ultrastructure of LGR-1 and/or BC treated cells was observed by transmission electron microscopy; scale bar in the lower right corner. **(B)** Expression of ZO-1 in cells measured by immunofluorescence analysis; scale bar in the lower right corner. **(C)** and **(F)** Protein levels of ZO-1 and Occludin in MAC-T. **(D)** and **(G)** Relative protein level of ZO-1. **(E)** and **(H)** Relative protein level of Occludin. The data of the CONTROL group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA. Data are means $\pm$ SEM of 3 independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001.

Figure 3

LGR-1 attenuated *B. cereus*-induced NLRP3 inflammasome activity. The MAC-T cells were treated with BC (MOI = 5) and/or LGR-1 (MOI = 100) for 3 h. (A) and (B) Protein levels of NLRP3, ASC, Caspase1 p20, GSDMD p30. (C) IL-1 β and IL-18 mRNA level. Expression of NLRP3, ASC, GSDMD and Caspase1 in cells measured by immunofluorescence analysis; scale bar in the lower right corner. (D) NLRP3. (E) ASC. (F) GSDMD. (G) Caspase1. The data of the CONTROL group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA. Data are means $\pm$ SEM of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 4

***B. cereus*-induced NLRP3 inflammasome activity requires K⁺ efflux.** 3 h after infection with BC (MOI = 5) or LPS (500 ng/mL) + ATP (5 mM) in the absence (–) or presence (+) of 50 mM KCl (+) or at increasing concentrations of KCl (5, 25, 50 and 75 mM). (A) IL-1 β mRNA level. (B) IL-18 mRNA level. (C) Cell death in MAC-T. The data of the CONTROL group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA. Data are means $\pm$ SEM of 3 independent experiments. * P < 0.05 and *** P < 0.001.

Figure 5

LGR-1 attenuated LPS + ATP-induced NLRP3 inflammasome activity. The MAC-T cells were treated with LPS (500 ng/mL) + ATP (5 mM) and/or LGR-1 (MOI = 100). **(A)** Protein levels of NLRP3 and Caspase1 p20. **(B)** Protein levels of GSDMD p30. **(C)** IL-1β mRNA level. **(D)** IL-18 mRNA level. **(E)** Relative protein level of NLRP3. **(F)** Relative protein level of Caspase1 p20. **(G)** Relative protein level of GSDMD p30. The data of the CONTROL group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA. Data are means ± SEM of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 6

BC induced NLRP3 inflammasome activity. The MAC-T cells were treated with BC (MOI = 5) and/or MCC950 (100 nM). **(A)** Protein levels of NLRP3, Caspase1 p20, GSDMD p30. **(B)** IL-1 β mRNA level. **(C)** IL-18 mRNA level. **(D)** Relative protein level of NLRP3. **(E)** Relative protein level of Caspase1 p20. **(F)** Relative protein level of GSDMD p30. The data of the CONTROL group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA. Data are means $\pm$ SEM of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 7

Knockdown of NLRP3 attenuated inflammatory responses. MAC-Ts were transfected with NLRP3 siRNA for 5 h and/or treated with BC (MOI = 5) and/or LGR-1 (MOI = 100) for another 3 h. **(A)** NLRP3 mRNA level. **(B)** The protein level of NLRP3. **(C)** Relative protein level of NLRP3. **(D)** The protein level of Caspase1 p20. **(E)** IL-1 β mRNA level. **(F)** IL-18 mRNA level. **(G)** Cell death in MAC-T. **(H)** Relative protein level of Caspase1 p20. In A and C, the data of the si-CONTROL group were used to normalize the data of each treatment group. Comparisons among groups were calculated using the t-test. Data are means $\pm$ SEM of 3 independent experiments. In E-H, the data of the CONTROL group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA. Data are means $\pm$ SEM of 3 independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001.

Figure 8

Schematic of the proposed model. LGR-1 can reduce the K⁺ efflux induced by BC, inhibit the NLRP3 inflammasome activity, decrease the expression of IL-1β and IL-18, reduce the production of active N-terminal fragment of GSDMD, and reduce pyroptosis and inflammatory response by protecting the intercellular tight junction. The solid black arrows indicate demonstrated effects, the dashed arrow

indicates the potential effects, the red lines indicate negative or protective effects, and the solid red arrows represent changes in expression.