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ORIGINAL PAPER

C. sakazakii activates AIM2 pathway accompanying with excessive ER stress response in mammalian mammary gland epithelium

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Abstract

Bovine mastitis is a common inflammatory disease caused by various factors. The main factor of mastitis is pathogenic microorganism infection, such as *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus*. *Cronobacter sakazakii* (*C. sakazakii*) is a newly discovered pathogenic bacteria in milk products, which seriously threat human health in recent years. At present, it has not been reported that the pathogenesis of mastitis is caused by *C. sakazakii*. This study investigated the inflammation of mammary gland epithelium, which was induced by *C. sakazakii* for the first time. We focused on bacterial isolation, histological observation, AIM2 inflammasome pathways, endoplasmic reticulum stress, and apoptosis. The results showed that *C. sakazakii*–induced inflammation caused damage of tissue, significantly increased the production of pro-inflammatory cytokines (including TNF-α, IL-1β, and IL-6), activated the AIM2 inflammasome pathway (increased the expression of AIM2 and cleaved IL-1β), and induced endoplasmic reticulum stress (increased the expression of ERdj4, Chop, Grp78) and apoptosis (increased the ratio of Bax/Bcl-2, a marker of apoptosis). In conclusion, it is suggested that it maybe inhibite AIM2 inflammasome pathways and alleviate endoplasmic reticulum stress (ER stress) against the *C. sakazakii*–induced inflammation.

Keywords *Cronobacter sakazakii* · AIM2 inflammasome · ER stress · Apoptosis

Introduction

Mastitis is a common disease, which causes a decline in milk yield and milk quality and seriously hinders the development of dairy industry, even harms to human health (Zheng et al. 2016). The etiologies of bovine mastitis are complicated, including bacteria, fungi, mycoplasma, and viruses (Filioussis et al. 2019). There were abundant studies of the mechanism of mastitis, mainly focused on *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Streptococcus agalactiae* (*S. agalactiae*), and *Streptococcus* (Haapala et al. 2018). Yet *C. sakazakii*, newly discovered in recent years and a kind of pathogenic bacteria in milk products, is an important emerging neonatal pathogen. It is associated with outbreaks of meningitis, sepsis, and necrotizing enterocolitis (Mullane et al. 2007), with a high mortality rate of 50–80% (Healy et al. 2010). It has not been reported whether *C. sakazakii* can cause caw mastitis. So far, the mechanism of *C. sakazakii*–induced inflammation remains poorly understand. Hence, it is urgent to understand its pathogenesis. In this report, we used *C. sakazakii* to construct inflammatory models in vivo and in vitro.

Inflammasome is a multiprotein complex composed of a sensor-receptor protein, a specular protein (ASC) associated with apoptosis, and a cysteine protease caspase-1, which activation promotes the release of mature IL-1β and IL-18 which play an indispensable role in defense factor in the innate immune response against invading microorganisms (Kovacs and Miao 2017). The NLR family consists of several receptors (Costa Franco et al. 2019). AIM2 is identified as the receptor involved in inflammasome activation, which responds to the recognition of cytoplasmic DNA during bacterial infections (Song et al. 2019). AIM2 can be activated by pathogens; its activation, Caspase-1 precursors, promotes releases of mature caspase1 and pro-inflammatory cytokine IL-1β, which result in inflammation (Schroder and Tschopp 2010; Wu et al. 2019).

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Endoplasmic reticulum (ER) is an organelle with many vital cellular functions (Schwarz and Blower 2016). ER stress maybe induced by physiological and pathological stimuli, for instance inflammation, oxidative stress, hypoxia, disturbance of calcium homeostasis, increase of protein expression, and subsequent accumulation of unfolded protein (Minamino and Komuro 2010), which plays a crucial role in many diseases, such as inflammatory bowel disease (Cai et al. 2019), diabetes (Sato et al. 2015), cardiovascular diseases (Lebeaupin et al. 2018), non-alcoholic fatty liver disease (Zhang et al. 2019), viral infection (Clarke et al. 2014), and immune-related disorders (Eggleton et al. 2017). Cellular response relies on the level of ER stress. The cells can recover and adapt to the ER stress when ER stress is milder. On the contrary, ER stress is prolonged or too severe; these mechanisms will imbalance protein homeostasis, leading to autophagy. If the stress is not alleviated, apoptosis may occur (Lee et al. 2019). Numerous studies have shown that apoptosis is essential for immune defense, which is associated with many diseases (Peterson et al. 2017). In addition, certain pathogens are capable of increasing epithelial apoptosis, thereby leading to onset of diseases (Wu et al. 2017). However, an imbalance between normal rates of apoptosis and restitution may result in destroying the cell barrier allowing for bacterial invasion and ultimately leading to the development of systemic sepsis (Hunter et al. 2008).

Materials and methods

Milk sample collection

A total of 20 samples of milk, including ten normal milk samples and ten mastitis milk samples, were collected from Yonghao Animal Husbandry Company, Jiangsu, China.

Bacterial strains

C. sakazakii is a newly discovered pathogenic bacterium in milk products. *C. sakazakii*, a mastitis pathogen, was isolated from the fresh milk with high somatic cell count and used for the intramammary inoculum to establish mice models of mastitis, which was used in this study.

Bacterial culture was performed in *C. sakazakii* chromogenic medium and incubated at 37 °C for 24 h. Overnight cultures were used for the intramammary injection in mice. *C. sakazakii* were properly diluted in PBS to achieve the desired concentration (2×10^8 CFU/mL). The bacterial inoculum size was determined by serially diluting the culture and pour plating the dilutions on common nutrition agar.

Cell culture and treatment

Bovine mammary epithelial cells (BMECs) were donated by professor Yuping Sun. Cells were cultured in DMEM containing 10% FBS at 37 °C in a humidified incubator under 5% CO_2. The medium was changed every 2 days. Cells were treated for 24 h by 50 μL (10^8 CFU/mL) inactivated bacteria which were diluted in PBS.

Animals

A total of 30 female and 10 male C57BL/6J mice (6–8 weeks old, weighing20–25 g) were kept in our own SPF mice house. The female and male mice cohabited at a 3 female to 1 male ratio for conception. They were housed separately after pregnancy until 5 to 7 days after calving. Lactating female mice were raised in a room maintained at 24 °C ± 1 °C with 40–80% humidity and were allowed access to food and water ad libitum. Feeding and management of experimental animals shall be executed in accordance with the relevant regulations of the national institutes of health on the use of experimental animals.

The procedures involving animals were approved by the Animal Welfare Committee of Nanjing Agricultural University, Nanjing, China, approval No. 20190205. And all animal experiments were carried out in strict accordance with the guidelines and rules.

Mouse model of mastitis

Female mice were separated into two groups at random: *C. sakazakii* group, treatment with *C. sakazakii*; PBS group, treatment with sterile PBS after postpartum 7 days. The pups were removed 2 h before the experiment. The female mice were anesthetized with ether and disinfected. The fourth pair of milk areas with 75% alcohol fully exposes the breast. The nipple was fixed in the left, and a 100 μL microinjector needle was slowly inserted into the milk ducts with the right hand, and *C. sakazakii* group was injected 50 μL of a certain concentration of bacterium into the mammary gland, while PBS group was injected with 50 μL PBS.

All mice were sacrificed by cervical dislocation after 24-h infection. The L4 (the left fourth mammary gland) and R4 (the right fourth mammary gland) mammary glands of each animal were sterile collected for follow-up analysis.

Histopathological analysis

The mammary gland samples of *C. sakazakii* group and PBS group were collected and fixed in 10% neutral buffered formalin for about 48–72 h, then embedded in paraffin, sectioned, and stained by hematoxylin and eosin (H&E), and the pathological changes were observed by microscopy.

Total RNA isolation and qRT-PCR analysis

The tissue of appropriate size was ground into a powder, and RNA was extracted by using Trizol (Invitrogen) following the manufacturer's instructions. The RNA was reverse-transcribed into cDNA in accordance with the Reverse Transcription System's instructions (Takara) according to the manufacturer's instructions. Real-time quantitative PCR was performed on an ABI 7500 RT-PCR instrument by using 2X SYBR Green (Vazyme, Q111-02) and the appropriate primers. According to the GenBank sequence, the primer sequence of the target genes and the *β-actin* gene were designed using the software PrimerPremier5.0 (Table 1).

Western blot analysis

The tissue (50-mg tissue) was ground into a powder in lysis buffer (mixture protein lysate and proteinase inhibitor as 1:9). The lysate was centrifuged at 13,000*g* for 20 min at 4 °C, and the supernatant was collected into a new centrifuge tube, and the supernatant was quantified using a BCA Protein Assay Kit (Beyotime Biotechnology Co.). Equal amounts of protein from each sample were mixed with SDS-PAGE loading buffer, then placed into metal incubator for 95 °C 10 min and preserved at 4 °C. Proteins were separated by SDS-PAGE and transferred to the PVDF membrane. After protein transfer, membranes were blocked for 1 h with 5% no-fat dried milk in TBST. The membranes were incubated overnight at 4 °C with rabbit monoclonal. The antibodies used in this study include the following: GAPDH (CST, Boston, MA), Tublin (CST, Boston, MA), Bax (Proteintech Co LTD, Wuhan, Hubei, China), Bcl-2 (Proteintech Co LTD, Wuhan, Hubei, China), AIM2 (CST, Boston, MA), and Cleaved IL-1β (CST, Boston, MA). Finally, membranes were processed using an enhanced chemiluminescence solution detection system (Amersham, Piscataway, NJ). Densitometry was performed by using the ImageJ software (National Institutes of Health, Bethesda, MD).

Table 1 The primer used for qRT-PCR analysis

Gene	Primer sequence
TNF-α	ATGAGCACAGAAAGCATGATC
	TACAGGCTTGTCACTCGAATT
IL-1β	ATGGCAACTGTTCCTGAACTCAACT
	CAGGACAGGTATAGATTCTT TCCTTT
IL-6	CTCCCAACAGACCTGTCTATAC
	CCATTGCACAACTCTTTTCTCA
Grp78	ACTTGGGGACCACCTATTCCT
	ATCGCCAATCAGACGCTCC
Chop	GCATGAAGGAGAAGGAGCAG
	ATGGTGCTGGGTACACTTCC
ERdj4	TAAAAGCCCTGATGCTGAAGC
	TCCGACTATTGGCATCCGA
Bax	TGCAGAGGATGATTGCTGAC
	GATCAGCTCGGGCACTTTAG
Bcl-2	ACTGTGTTAACTCCTGCCCG
	GCAGCAAGCTACTCAGACGA
Actin	GACCTCTATGCCAACACAGT
	AGTACTTGCGCTCAGGAGGA

Statistical analysis

Statistical analyses were performed by *t* test with GraphPad Prism version 7.0 (GraphPad Sofware, San Diego, CA, USA). Data were presented as mean ± the standard deviation (SD). $P < 0.05$ were considered statistically significant.

Results

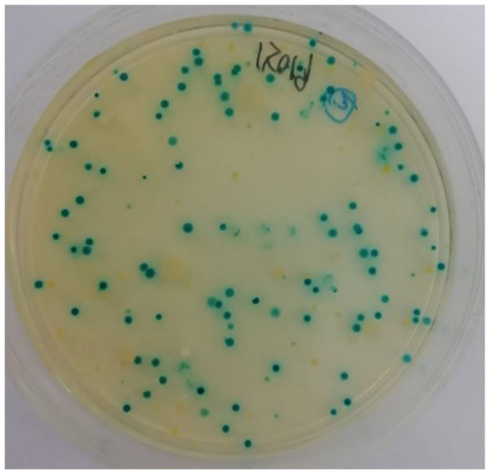
Isolation and identification of *C. sakazakii*

C. sakazakii is a pathogenic bacterium frequently detected in dairy products in recent years. We isolated five strains from 54 colonies by 16sRNA sequencing, including *C. sakazakii*, *Pantoea rwandensis*, *Escherichia coli*, *Staphylococcus aureus*, and *Aerococcus vitidans*. In order to further confirm, we used the characteristic of *C. sakazakii* showing blue colonies in *C. sakazakii* chromogenic medium (Fig. 1a). PCR assay was developed based on α-1, 4-glusidase gene. The ban size was 673 bp (Fig. 1b), and PCR products were recovered and purified, which were sent to sequencing (Sangon Biotech, Shanghai, China). All the obtained sequences were submitted to the GenBank. The results showed that the homology of the target fragment of α-1, 4-glucosidase gene (673 bp) was 96% (Fig. 1c).

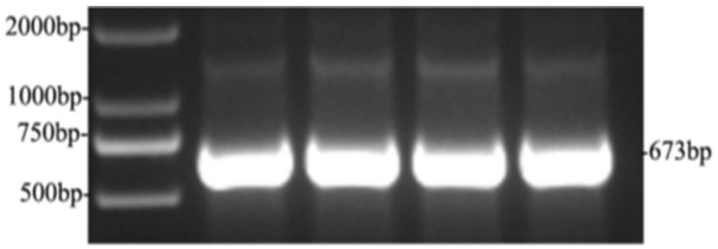
C. sakazakii–induced histopathological impairment and increased pro-inflammatory cytokine levels of the mammary gland in mice

Mice were sacrificed 24 h later. After dissection, we found that the tissues of the fourth breast area, injected with bacterium, were thickened and congested, while the first and second breast areas, injected with PBS, were full of milk (Fig. 2a). To test whether the mouse mastitis model was successfully constructed, the mammary gland was collected, sectioned, and stained with H&E. The results showed that there were no pathological injuries of mammary gland from the PBS group mice (Fig. 2b). Compared with the PBS group, mammary gland from the *C. sakazakii* group presented severe pathological injuries, such as tissue congestion and edema, interstitial thickening, and a large number of inflammatory cells

a



b



c

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Cronobacter sakazakii strain ATCC 29544, complete genome	1074	1074	98%	0.0	96.75%	NZ_CP011047.1
Franconibacter pulveris DSM 19144 Q370DRAFT_scaffold00001.1, whole genome shotgun sequence	625	625	98%	8e-176	84.23%	NZ_KK211221.1
Nigerium massiliense strain SIT5, whole genome shotgun sequence	110	110	20%	1e-20	81.34%	NZ_CCYM01000003.1
Actinoplanes philippinensis strain DSM 43019, whole genome shotgun sequence	82.4	82.4	21%	2e-12	77.62%	NZ_FONV01000005.1
Cellulomonas marina strain CGMCC 4.6945, whole genome shotgun sequence	76.8	76.8	16%	1e-10	79.44%	NZ_FOKA01000003.1

Fig. 1 Isolation and identification of *C. sakazakii*. Identification of *C. sakazakii*: **a** *C. sakazakii* chromogenic medium is a selective medium. *C. sakazakii* showed green colonies. **b** PCR assay was developed based on α-1, 4-glusidase genes, which were positive (673 bp). **c** Sequencing: the homology of the target fragment of α-1, 4-glucosidase gene (673 bp) was 96%

infiltration. Most of the epithelial cells were necrosis, exfoliation, disintegration, or even disappearance. Neutrophils were scattered in mammary gland acinus, which were necrosis and disintegration (Fig. 2c). Pro-inflammatory cytokines play important roles in the development of inflammation to further examine the degree of inflammation in mice with mastitis induced by *C. sakazakii*. The production of the pro-inflammatory cytokines, including *TNF-α*, *IL-1β*, and *IL-6*, was measured by qRT-PCR. It showed that *C. sakazakii* group markedly increased the mRNA levels of *TNF-α* (Fig. 2d), *IL-1β* (Fig. 2e), and *IL-6* (Fig. 2f) compared with the PBS group.

C. sakazakii increased the expression of AIM2 and cleaved IL-1β in mice and in BMECs

Studies have shown that AIM2 is required for the release of IL-1β in the macrophage infected with virulent *B. abortus* (Gomes et al. 2013). AIM2 can be activated by pathogens; its activation promotes the cleavage of caspase-1 and releases the mature pro-inflammatory cytokine IL-1β, which induce inflammation (Gong et al. 2019). To study the role of AIM2 in the *C. sakazakii*–induced inflammation in mice and BMECs, the expression of AIM2 and cleaved IL-1β were detected by western blot. The result showed that the *C. sakazakii* group significantly increased the expression of AIM2 and cleaved IL-1β protein in mice and BMECs (Fig. 3 a and c). The quantitative analysis of protein further confirmed that the *C. sakazakii* group significantly increased in the expression of AIM2 and cleaved IL-1β comparing with the PBS group ($P < 0.0001$, Fig. 3 b and d).

C. sakazakii–induced ER stress in mice and in BMECs

Recently, substantial evidences have proved that inflammatory response is related to ER stress, and pro-inflammatory cytokines could induce excessive ER stress response (Brenjian et al. 2019) to assess whether *C. sakazakii* stimulated ER stress in mice and in BMECs. The expression of ER stress marker was detected by western blot. The result showed that *C. sakazakii*–injected significantly increased the Grp78, a member of the HSP protein family, which was found in the ER that was upregulated after ER stress (Zheng et al. 2019) and C/EBP homologous protein (Chop) (Pejman et al. 2019), a pro-apoptotic transcription factor that was overexpressed following the disruption of ER homeostasis, compared with the PBS group in mice (Fig. 4 a and c). Further confirmed *C. sakazakii* group resulted in a significant increase in the expression of AIM2 and cleaved IL-1β compared with the PBS group ($P < 0.0001$, Fig. 4 b and d). We also detected the mRNA expression of *ERdj4*, a BiP cochaperone that was

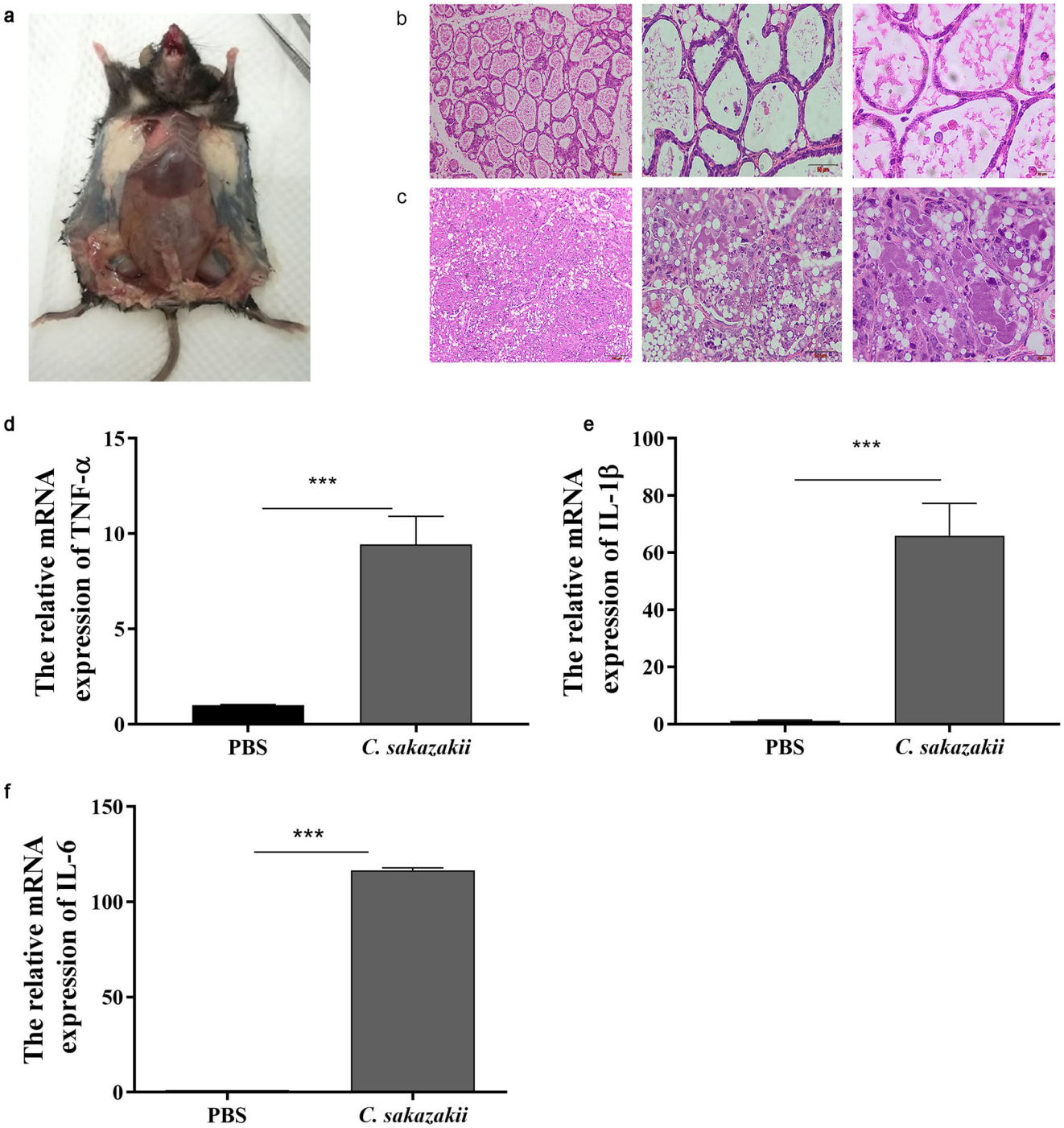


Fig. 2 *C. sakazakii* induced histopathological impairment and increased pro-inflammatory cytokines levels of mammary gland in mice. **a** A mouse model of mastitis: the fourth pair of mammary glands were injected with *C. sakazakii* and the first and second pairs of mammary glands were injected with PBS and dissected. Mouse was dissected 24 h after infection. **b** HE staining of mammary glands histology: each nipple was injected with 100-μL *C. sakazakii* fluid. **c** HE staining of mammary glands histology: each nipple was injected with 100-μL PBS. The mRNA level of *TNF-α* (**d**), *IL-1β* (**e**), *IL-6* (**f**), and relative mRNA level of *β-actin* were determined by qRT-PCR. Values are presented as means ± the standard deviation (SD) ($n \geq 3$) (***$P<0.001$)

upregulated after ER stress (Fritz et al. 2014), *Chop*, and glucose-regulated protein 78 (*Grp78*) by qRT-PCR. The result showed that *C. sakazakii*–injected significantly increased the mRNA expression of *ERdj4*, *Chop*, and *Grp78* (Fig. 4e) compared with the PBS group in mice. Moreover, BMECs were treated with bacterium and the result showed that the expression of ERdj4, *Chop*, and Grp78 increased compared with the control group (Fig. 4).

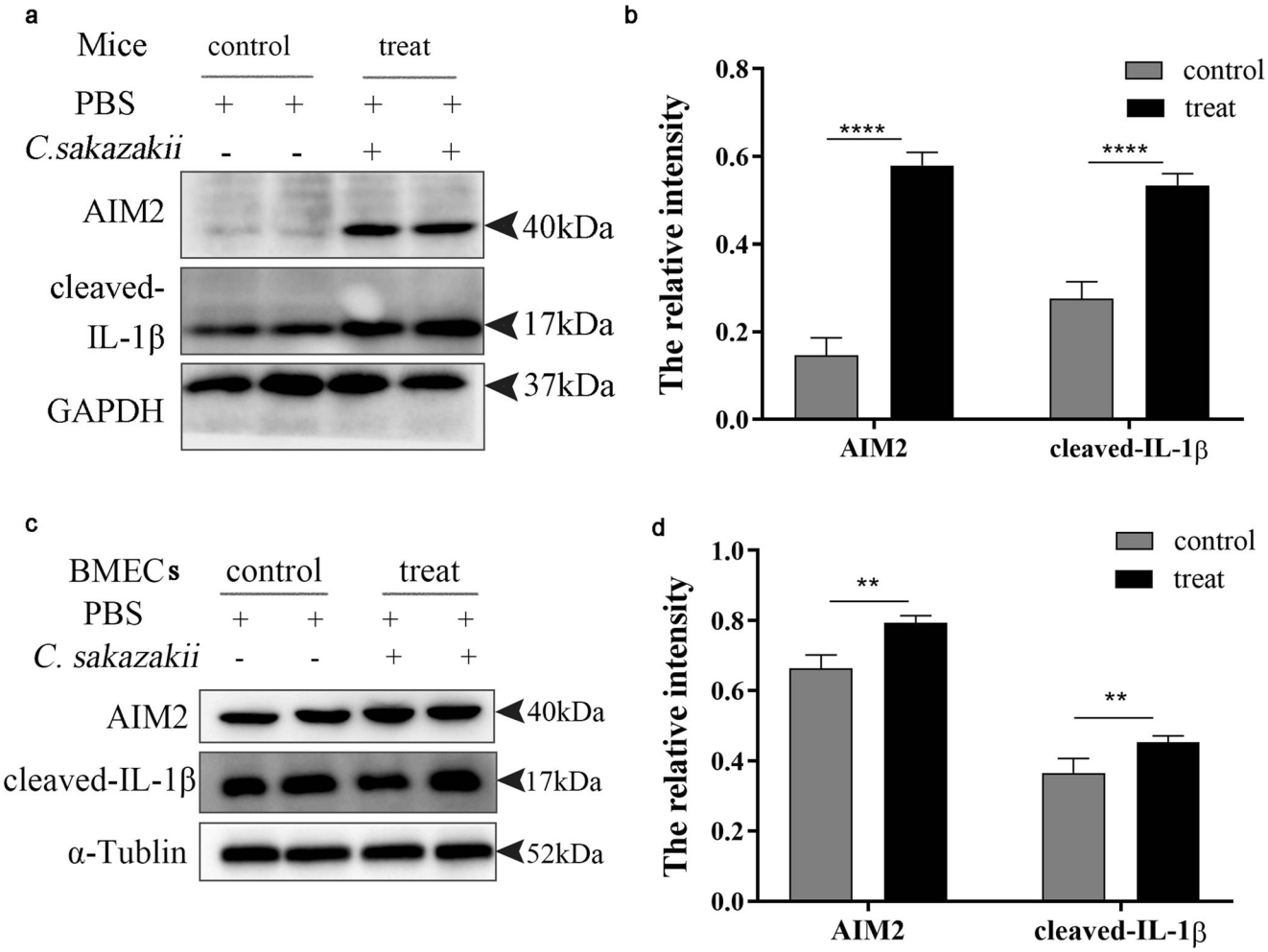


Fig. 3 *C. sakazakii* increased the expression of AIM2 and cleaved IL-1β in vivo and vitro. The mammary gland tissues of each group ($n \geq 3$) were obtained at 24 h after *C. sakazakii* and PBS infection. **a**, **c** AIM2 protein and cleaved IL-1β protein were detected by western blot in mice and in BMECs. **b**, **d** The relative AIM2 protein and cleaved IL-1β protein levels were quantified by ImageJ and normalized to α-Tublin. Data are presented as means ± the standard deviation (SD) ($n \geq 3$) (**P<0.01, ****P<0.0001)

C. sakazakii–induced apoptosis in mice and BMECs

Apoptosis interacted with many vital processes such as inflammation (Rathinam et al. 2010). Therefore, we examined the apoptosis process by western blot to investigate whether the inflammation caused by *C. sakazakii* activated apoptosis. The expression of Bax, a pro-apoptotic protein (Bialuk et al. 2019), and Bcl-2, antiapoptotic protein (Bialuk et al. 2019), was detected by western blot in mice and BMECs. As shown in Fig. 5, the *C. sakazakii* group significantly increased the level of Bax and decreased the expression of Bcl-2 in mice and BMECs (Fig. 5 a and c). The further confirmed *C. sakazakii* group resulted in a significant increase in the ratio of Bax/Bcl-2 ($P < 0.001$) compared with the PBS group (Fig. 5 b and d). Moreover, we detected the mRNA expression of *Bax* and *Bcl-2* by qRT-PCR. It showed that the *C. sakazakii* treatment group significantly increased the mRNA level of *Bax* ($P < 0.05$) and decreased the expression of *Bcl-2* ($P < 0.01$) (Fig. 5e), and the ratio of *Bax/Bcl-2* significantly increased in mice (Fig. 5f).

Discussion

Mastitis seriously affects the milk yield and quality of dairy cows and seriously hinders the development of dairy industry, even threats to human health. In recent years, *C. sakazakii* was frequently detected in certain infant formulas made with milk (Chen et al. 2019). With the increased with the number of using infant formula, *C. sakazakii*–related infections have substantially increased. It has been reported that necrotizing enterocolitis may be associated with *C. sakazakii*. At present, the mechanism of *C. sakazakii*–induced mastitis inflammation has not been reported. We were the first to used *C. sakazakii* to construct inflammatory models to study the mastitis in vivo and in vitro. Our results were consistent with the previous

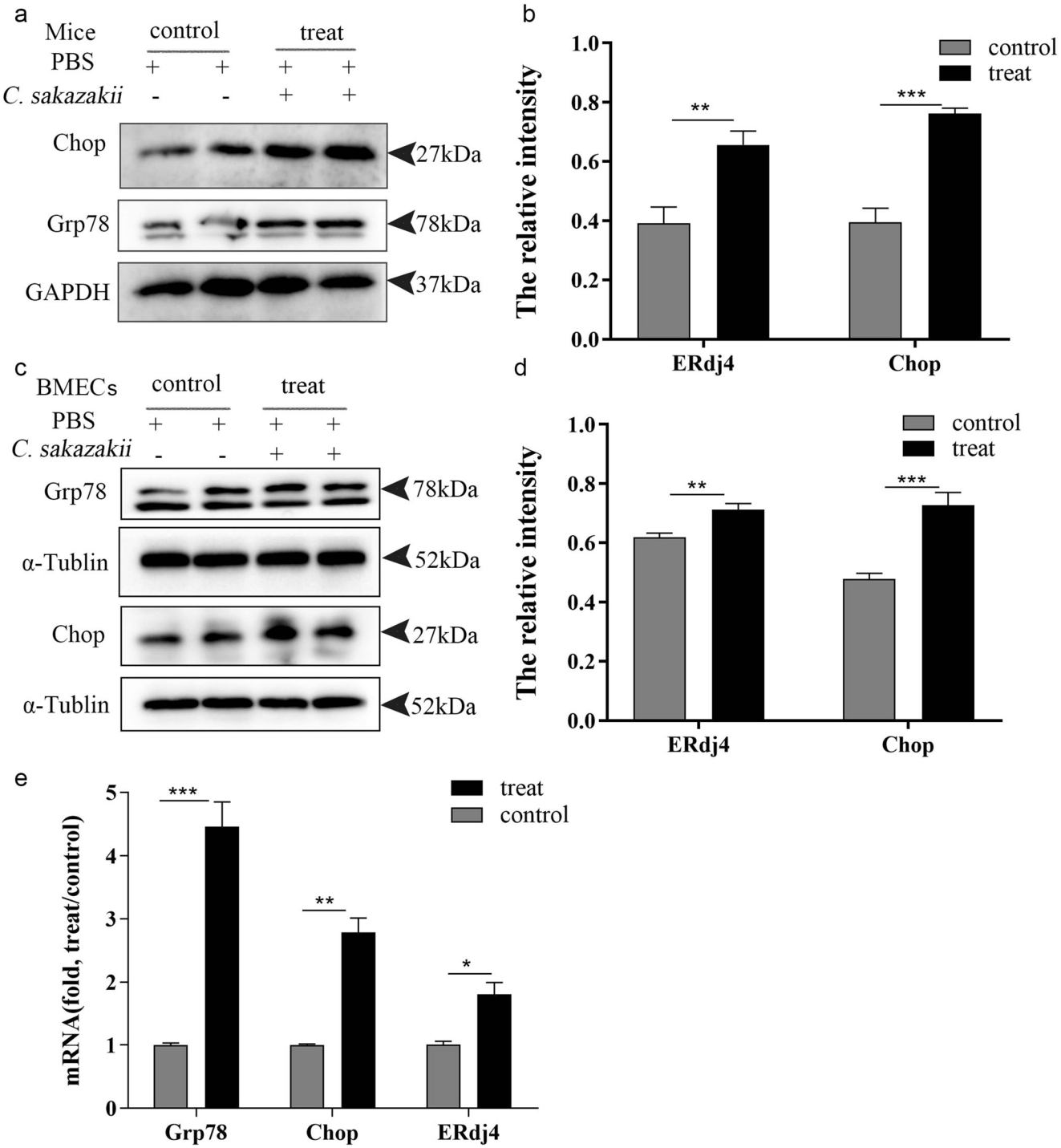


Fig. 4 *C. sakazakii* induced endoplasmic reticulum (ER) stress in mouse models and in BMECs. The mammary gland tissues of each group ($n \geq 3$) were obtained at 24 h after *C. sakazakii* and PBS infection. **a**, **c** Grp78 protein and Chop protein were detected by western blot in mice and in BMECs. **b**, **d** The relative Grp78 protein and Chop protein levels were quantified by ImageJ and normalized to α-Tublin and GAPDH in mice and BMECs. **e** The mRNA level of *Grp78*, *Chop*, *ERdj4*, and relative mRNA level of *β-actin* were determined by qRT-PCR in mice. Data are presented as means ± the standard deviation (SD) ($n \geq 3$) (*P<0.05, **P<0.01, ***P<0.001)

studies which LPS induced mastitis in BMECs that the production of TNF-α increased significantly, which was a crucial immunoregulatory cytokine that could trigger a series of immune responses and subsequently accelerate the production of other cytokines (Li et al. 2019). It has not been reported the *C. sakazakii* cause caw mastitis. In this study, we used the desired concentration (2×10^8 CFU/mL) to construct the mouse mastitis model. We found that mammary gland from

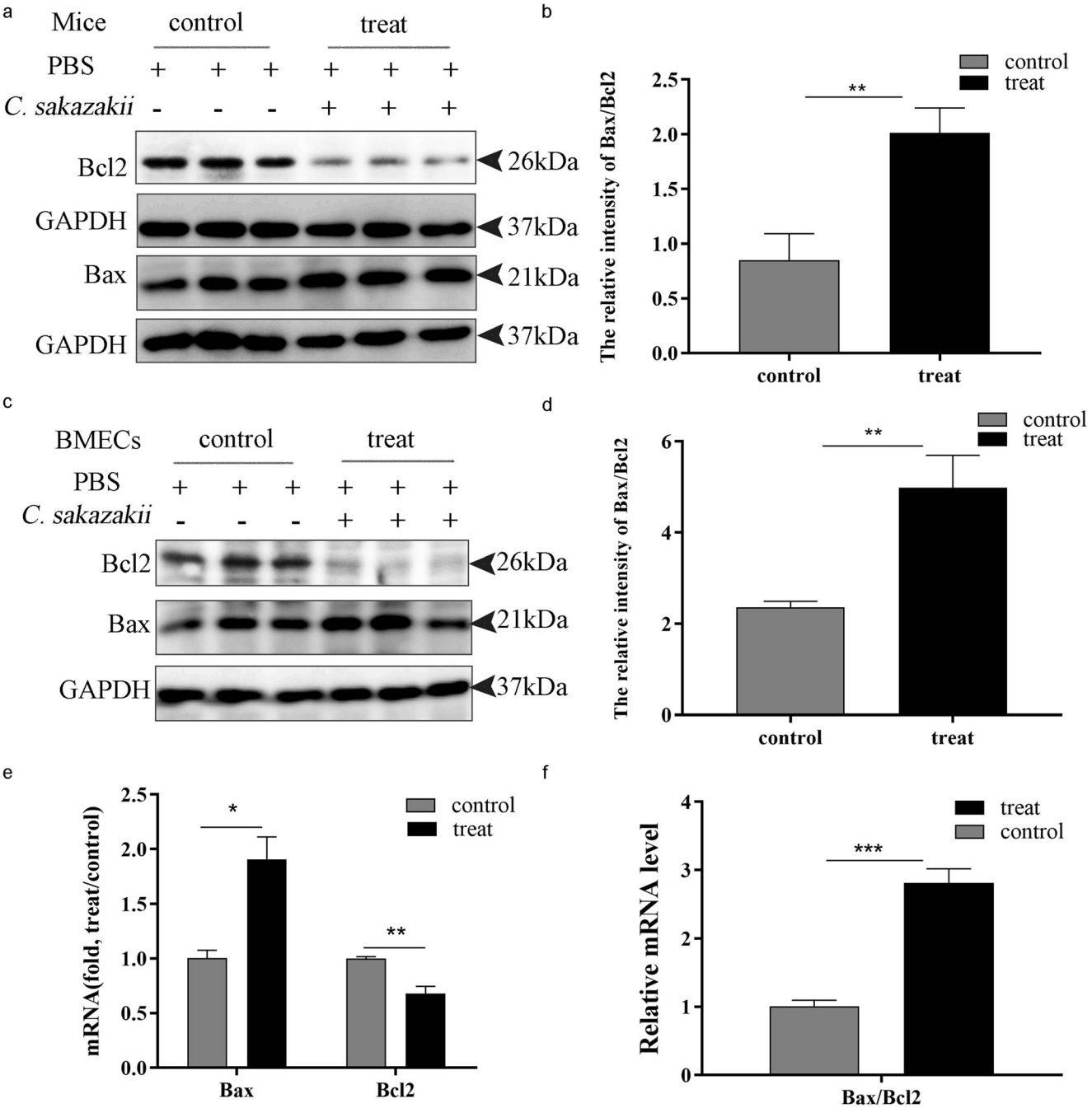


Fig. 5 *C. sakazakii* induced apoptosis in mouse model and in bovine mammary epithelial cells. The mammary gland tissues of each group ($n \geq 3$) were obtained at 24 h after *C. sakazakii* and PBS infection. **a**, **c** Bcl-2 protein and Bax protein were detected by western blot in mice and in BMECs. **b**, **d** The ratio of Bax/Bcl-2 protein was analyzed in mice and BMECs. **e** The mRNA level of *Bcl-2*, *Bax*, and relative mRNA level of *β-actin* were determined by qRT-PCR in mice. **f** The ratio mRNA level of *Bcl-2*/*Bax* and relative mRNA level of *β-actin* were determined by qRT-PCR in mice. Data are presented as means ± the standard deviation (SD) ($n \geq 3$) (*$P<0.05$, **$P<0.01$, ***$P<0.001$)

the *C. sakazakii* group presented severe pathological injuries compared with the PBS group, and the production of IL-6 and IL-1β were significantly increased in the mammary gland tissues treated by *C. sakazakii* than treated by PBS.

Studies have shown that AIM2 is required for the release of IL-1β in the macrophage infected with virulent *B. abortus* (Gomes et al. 2013). Moreover, *B. abortus* infection eliminated IL-1β in NLRP3-deficient macrophages, but did not affect IL-1β production in AIM2-deficient macrophages (Bronner et al. 2015). It has been shown that nigericin and ATP-stimulated total bone marrow cells lead to the NLRP3 inflammasome activation, and *Salmonella*-stimulated bone marrow neutrophil leads to the NLRC4 inflammasome activation, which promotes

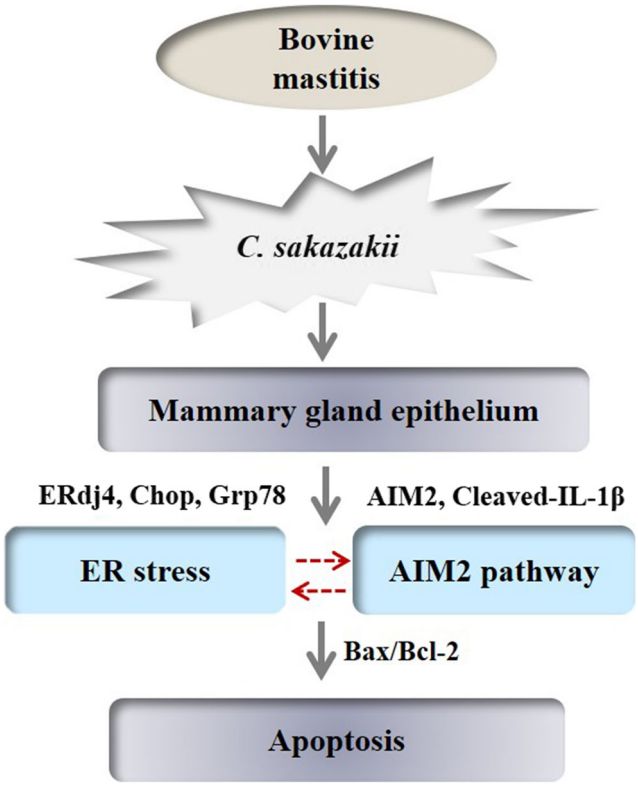


Fig. 6 *C. sakazakii*–stimulated mammalian mammary gland epithelium induced apoptosis via activating AIM2 pathway and excessive ER stress response. *E. sakazakii* was isolated from bovine mastitis milk sample, which was used to infected mice and BMECs. *E. sakazakii*–infected mammary gland epithelium activated AIM2 inflammasome and induced excessive endoplasmic reticulum stress response. However, there has been no evidence to support the relationship between AIM2 inflammasome activation and endoplasmic reticulum stress. Finally, apoptosis occurred in mammary epithelial cells

the maturation of IL-1β (Chen et al. 2014; Mankan et al. 2012). Our results were consistent with the previous studies that AIM2 is necessary for the release of IL-1β during macrophage infection; we revealed that AIM2 and cleaved IL-1β increased in the *C. sakazakii* treatment group compared with the PBS group. We then reasoned that *C. sakazakii* activated AIM2 inflammasome pathway. Thus, it is possible to inhibit AIM2 inflammasome pathways to treat *C. sakazakii*–induced inflammation.

ER stress is associated with the activation of inflammation (Bronner et al. 2015). ER is a large membranous organelle with a series of functions, such as folding newly synthesized proteins, maintenance of calcium homeostasis and phospholipid synthesis, and regulating intracellular signaling pathways (Kleizen and Braakman 2004). Subversion of ER function is a feature shared by multiple intracellular bacteria and viruses, and this disruption of cellular function activates pathways of the unfolded protein response (UPR) (English et al. 2017). Substantial evidences have proved that inflammatory response is related to ER stress, and pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and interleukin 8 (IL-8), are closely related to the unfolded protein response (UPR) (Li et al. 2005). Our results showed that the *C. sakazakii* treatment group increased the expression of ERdj4, Chop, and Grp78 compared with the PBS group. Chop is a transcription factor, which is induced in response to physiological changes in the cell, including ER stress, DNA damage, nutrient deprivation, hypoxia, and viral and fungal infection (Kleizen and Braakman 2004; Li et al. 2014). Our research indicated that *C. sakazakii* induces excessive ER stress response.

Excessive ER stress results in apoptosis. Therefore, we further investigated the cell apoptosis. Numerous studies have shown that apoptosis is crucial for immune defense and associated with many diseases (Namgaladze et al. 2019). Moreover, certain pathogens are capable of increasing epithelial apoptosis, thereby contributing to disease pathogenesis (Ruan et al. 2016). However, when there is an imbalance between normal rates of apoptosis and restitution, this may result in decreased barrier integrity allowing for bacterial translocation and the development of systemic sepsis (Hunter et al. 2008). In agreement with this concept, our studies showed that the *C. sakazakii* treatment group significantly increased the expression of the Bax/Bcl2 than PBS group, which demonstrated that the *C. sakazakii* treatment group was significant apoptosis in BMECs and in mice.

Conclusion

We separated C. sakazakii from the fresh milk, which was used to construct the model of inflammation. *C. sakazakii*–induced inflammation significantly increased the expression of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, activated AIM2 inflammasome, and induced excessive ER stress response and apoptosis. However, there has been no evidence to support the relationship between AIM2 inflammasome activation and ER stress (Fig. 6). These data emphasized that *C. sakazakii* induced mammary epithelial cell apoptosis and stimulated the expression of inflammatory cytokines via AIM2 inflammatory activation and ER stress in vivo and in vitro, leading to tissue damage. Further understanding the role of *C. sakazakii* in the pathogenesis of mastitis will provide clues for developing new therapeutic strategies to prevent this disease.

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