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Lactobacillus acidophilus S-layer protein-mediated inhibition of *Salmonella*-induced apoptosis in Caco-2 cells

Pengcheng Li, Yinyan Yin, Qinghua Yu, Qian Yang *

College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China

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ABSTRACT

Surface layer (S-layer) proteins are crystalline arrays of proteinaceous subunits present as the outermost component of the cell wall in several *Lactobacillus* species. The underlying mechanism for how S-layer proteins inhibit pathogen infections remains unclear. To gain insights into the mechanism of the antimicrobial activity of *Lactobacillus* S-layer proteins, we examined how *Lactobacillus* S-layer proteins impact *Salmonella* Typhimurium-induced apoptosis in vitro in Caco-2 human colon epithelial cells. When Caco-2 cells infected with *Salmonella* Typhimurium SL1344, we found that apoptosis was mediated by activation of caspase-3, but not caspase-1. When *Salmonella* Typhimurium SL1344 and S-layer proteins were coincubated simultaneously, Caco-2 cell apoptosis was markedly decreased and the cell damage was modified, as evaluated by flow cytometry and microscopy. Detailed analyses showed that the S-layer proteins inhibited the caspase-3 activity and activated the extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling pathway. Taken together, these findings suggest that *Lactobacillus* S-layer proteins protected against *Salmonella*-induced apoptosis through reduced caspase-3 activation. In addition, c*Salmonella*-induced apoptotic cell damage was modified by S-layer proteins through the ERK1/2 signaling pathway. This mechanism may represent a novel approach for antagonizing *Salmonella* infection.

1. Introduction

The intestinal mucosa is an important route of entry for microbial pathogens. Furthermore, intestinal epithelial cells are the initial sites for host interactions with invasive enteric pathogens. Normal apoptosis in response to bacterial infection may function to delete infected and damaged epithelial cells, and to restore epithelial cell growth regulation and epithelial integrity that are altered during the course of enteric infection [1]. However, human epithelial cells have been shown to undergo abnormal apoptosis, which is linked to a delayed apoptosis response, following infection with invasive enteric pathogens. For example, apoptosis is delayed for 12–18 h after *Salmonella* infection in human intestinal epithelial cell lines [1,2]. The delay in onset of epithelial cell apoptosis may be critical for some intracellular pathogens, since it provides sufficient time for proliferation and adaptation to the intracellular environment to cause a large amount of cell injury [2–4]. *Salmonella*, an important enteropathogen that causes diarrheal disease, can induce serious cell damage by this delayed apoptotic response [1,2].

During pathogen-induced apoptosis, some critical factors are activated such as caspase-1 and caspase-3. Caspase-1 is a convergence point of several signaling pathways that control inflammatory responses [5]. Some pathogens activate caspase-1, which leads to the processing and release of numerous factors, including the proinflammatory cytokines interleukin (IL)-1β and IL-18 [6–8]. Caspase-3 is a key executioner caspase in the proteolytic cascade that leads to apoptotic cell death, and cleaves a number of structural proteins during the execution phase of apoptosis [9]. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are important components of mitogen-activated protein kinase (MAPK) signaling pathways, which mainly mediate the recovery of epithelial damage after infection with pathogens [10].

In recent years, S-layer proteins of several *Lactobacillus* species have been shown to play roles in the antimicrobial activities [11,12]. The S-layer proteins of species such as *Lactobacillus helveticus*, *Lactobacillus crispatus* and *Lactobacillus kefir* are able to inhibit the infection of certain pathogens in host epithelial cells, including *Salmonella enteritidis* [13] and *Salmonella* Typhimurium [14]. However, the underlying antimicrobial mechanisms remain unclear.

It is an important component of infection that the pathogen hijacks the host's apoptotic pathway to facilitate its pathogenesis. The aspect of whether *Lactobacillus* S-layer proteins inhibit pathogen-induced cell apoptosis has not been reported to date. There-

* Corresponding author. Address: College of Veterinary Medicine, Nanjing Agricultural University, Weigang No. 1, Nanjing, Jiangsu 210095, China. Fax: +86 25 84398669.
E-mail address: zxbyq@njau.edu.cn (Q. Yang).

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fore, the aims of this study were to determine the potential of S-layer protein extracts from lactobacilli to inhibit epithelial cell apoptosis mediated by *Salmonella* Typhimurium and to investigate the possible antimicrobial mechanisms of S-layer proteins. The findings may help us to better understand how *Lactobacillus* S-layer proteins inhibit *Salmonella*-induced apoptosis in human intestinal epithelial cells, and provide a rationale for the use of these proteins as therapeutic and preventative agents.

2. Materials and methods

2.1. Bacterial strains

Salmonella Typhimurium strain SL1344 was a generous gift from Prof. Shulin Liu (Peking University, Beijing, China). *Lactobacillus acidophilus* ATCC 4356 was purchased from the China Committee for Culture Collection of Microorganisms. For Caco-2 cell infection, *Salmonella* Typhimurium was routinely grown in Luria–Bertani medium overnight. The bacteria were collected by centrifugation, washed with sterile phosphate-buffered saline (PBS, pH 7.4) and resuspended in tissue culture medium without antibiotics. *L. acidophilus* ATCC 4356 was grown in De Man–Rogosa–Sharpe static cultures with minimal aeration at 37 °C for 24 h and harvested by centrifugation.

2.2. Cell culture

The human colon epithelial cell line Caco-2, a model of mature enterocytes of the small intestine, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Caco-2 cells (passages 45–65) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 4.5 g/l D-glucose, 25 mM HEPES, 1% nonessential amino acids and 2 mM L-glutamine (Gibco, Carlsbad, CA, USA). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Caco-2 cells were cultured until they reached confluency (differentiated cells).

2.3. S-layer proteins

S-layer proteins were obtained from *L. acidophilus* ATCC 4356 as previously reported [14]. Briefly, S-layer proteins were extracted from *L. acidophilus* ATCC 4356 using 4.0 M guanidine hydrochloride [15] and purified by chromatography on an anion-exchange column (DE52; Whatman, Kent, UK).

2.4. Infection of Caco-2 cells by Salmonella Typhimurium SL1344

Caco-2 cells were seeded into 24-well or 6-well plates and grown to confluency. *Lactobacillus* S-layer proteins (100 μg/ml) and *Salmonella* Typhimurium (5×10^7 CFU/well) were added to the Caco-2 cell monolayers and coincubated for 1 h at 37 °C under 5% CO_2. Gentamicin (40 μg/ml) was then added to each well and the monolayers were further incubated for different times before testing. All assays were performed in triplicate on three consecutive cell passages.

2.5. Apoptosis assays

Apoptosis of Caco-2 cells were assessed with an Annexin V-FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were stained with annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) for analyses by flow cytometry (cells seeded into 6-well plates) or microscopy (cells differentiated on glass coverslips placed in 24-well plates). Early apoptotic cells showed positive staining for annexin V-FITC alone, while necrotic cells and late apoptotic cells showed positive staining for both annexin V-FITC and PI. Flow cytometry was performed using a FACSCanto II cytofluorimeter (Becton Dickinson, Mountain View, CA, USA) with excitation at 488 nm. Fluorescence emission by FITC was measured using a 530/30 bandpass filter while that of PI was measured using a 585/42 bandpass filter. In addition, the cells were observed under a fluorescence microscope (AXIOVERT; Carl Zeiss, Jena, Germany).

2.6. Caspase-1 and caspase-3 activity assays

The activity of caspase-1 was determined using a Caspase-1 Activity Kit (Beyotime Institute of Biotechnology, Haimen, China), which is based on the ability of caspase-1 to change acetyl-Tyr-Val-Ala-Asp p-nitroanilide (Ac-YVAD-pNA) into the yellow formazan product p-nitroaniline (pNA). Cell lysates were centrifuged at 12,000*g* for 10 min, and the protein concentrations were determined by the Bradford protein assay. Cellular extracts (30 μg of protein) were incubated in a 96-well microtiter plate with 20 ng of Ac-DEVD-pNA overnight at 37 °C. The absorbance values of pNA at 405 nm, OD_{405}, were measured using a 96-well plate reader (BioTek, Santa Barbara, CA, USA). An increase in the OD_{405} indicated activation of caspase-1.

The activity of caspase-3 was determined using a Caspase-3 Activity Kit (Beyotime Institute of Biotechnology), based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) into the yellow formazan product pNA. The same method described for the caspase-1 assay was used to determine the activation of caspase-3.

2.7. Western blot analysis

Caco-2 cells were washed twice with ice-cold PBS (pH 7.2–7.4) and lysed with RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 10 mM EDTA pH 8.0 dissolved in PBS) containing phosphatase and protease inhibitors (1 mM PMSF, 1 mM Na_3VaO_4, 25 mM NaF) on ice for 30 min. The cell lysates were centrifuged at 14,000*g* for 10 min at 4 °C and the protein concentrations were determined. Protein preparations from Caco-2 cells were separated by 4–12% SDS–PAGE, and transferred onto nitrocellulose membranes. The loaded nitrocellulose membranes were probed with an anti-phospho-ERK1/2 primary antibody at a dilution of 1:1000, followed by goat anti-rabbit IgG-HRP (Bioworld, Dublin, OH, USA) as a secondary antibody. The specific signals were visualized with the Super Signal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. The signals were recorded on films or with a Kodak Image Station 2000 MM Multi-Modal Imager (Eastman Kodak Company, Rochester, NY). The membranes were then stripped and reprobed with an anti-total ERK1/2 antibody. The autoradiograms were scanned and analyzed with Quantity One software (Bio-Rad, Hercules, CA, USA) to quantify the band densities. The mean density of the phospho-ERK1/2 band was divided by the mean density of the corresponding total ERK1/2 band to yield a normalized band density value.

2.8. Data analysis

The results were expressed as means ± SEM. ANOVA and an unpaired Student's *t*-test were employed to determine the statistical significance of differences among multiple groups.

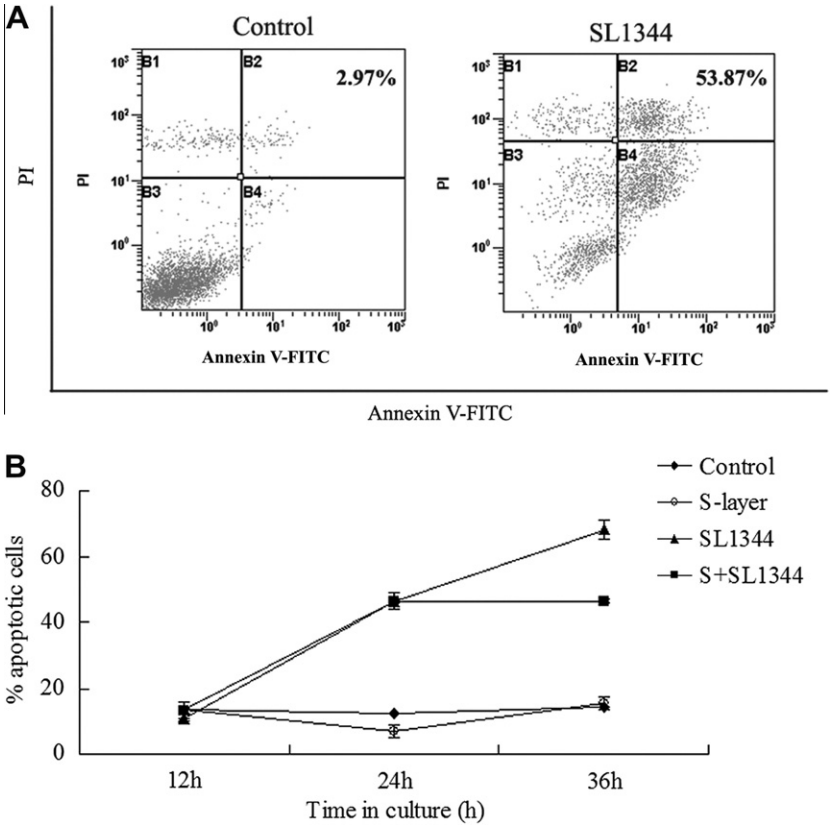

Fig. 1. Apoptosis of Caco-2 cells determined by flow cytometry after coincubation with *Salmonella* Typhimurium SL1344 and *Lactobacillus* S-layer proteins. The cell distribution was analyzed by Annexin V-FITC and PI uptake. (A) Apoptosis of Caco-2 cells was observed at 24 h after infection with *Salmonella* Typhimurium SL1344. The numbers indicate the percentages of cells present in the sums of the B2 and B4 areas. (B) Time courses of apoptosis in Caco-2 cells after *Salmonella* infection with S-layer proteins. The data shown are the means ± SEM of three independent experiments on three consecutive cell passages. The control cells received no treatment.

3. Results

3.1. Lactobacillus S-layer protein-mediated inhibition of Salmonella-induced apoptosis in Caco-2 cells

Flow cytometry revealed that apoptosis was induced in *Salmonella* Typhimurium SL1344-infected Caco-2 cells. As shown in Fig. 1A, the percentage of apoptotic cells was increased to 53.87 ± 2.93% after *Salmonella* infection compared with 2.97 ± 0.90% in uninfected control cells. In addition, apoptosis occurred as a relatively late event, since the commitment of Caco-2 cells to undergo apoptosis was delayed for 12 h after infection. The apoptotic cells were progressively and significantly increased in a time-dependent manner. S-layer proteins alone did not affect Caco-2 cell apoptosis. However, when Caco-2 cells infected with *Salmonella* were coincubated with the S-layer proteins for 36 h, the apoptotic cells were significantly decreased ($P < 0.01$; Fig. 1B).

Morphologic assessments of apoptosis by microscopy were well matched with the flow cytometry data. When Caco-2 cells were infected with *Salmonella* Typhimurium SL1344 for 24 h, many apoptotic cells and necrotic cells showed red and green fluorescence (Fig. 2A). Untreated Caco-2 cells or those treated with S-layer proteins alone showed little green fluorescence or no fluorescence, indicating the presence of few apoptotic cells. The S-layer proteins did not affect the change in *Salmonella*-induced apoptosis in Caco-2 cells up to 24 h. As shown in Fig. 2B, when Caco-2 cells were infected with *Salmonella* for 36 h, a large number of necrotic cells and some detached cells exhibiting strong red fluorescence were observed. However, S-layer proteins markedly inhibited the apoptosis-induced cell injury. The cells showed little fluorescence and were restored to their former compact condition.

3.2. Effects of S-layer proteins on caspase-1 activity

First, we analyzed whether *Salmonella* Typhimurium affected the activation of caspase-1 in epithelial Caco-2 cells, as assessed by cleavage of the caspase-3 substrate Ac-YVAD-pNA. After *Salmonella* Typhimurium SL1344 infection, the activation of caspase-1 was not altered significantly at 12 h and, interestingly, was decreased at 24 h (13.45 ± 0.57% reduction, $P < 0.05$) and 36 h (19.20 ± 0.78% reduction, $P < 0.01$) compared with uninfected cells (Fig. 3A). When Caco-2 cells treated with *Salmonella* and S-layer proteins simultaneously, the activation of caspase-1 was not changed significantly compared with *Salmonella* treatment alone at 12 h and 24 h, and was restored to the normal level at 36 h ($P < 0.01$). S-layer proteins alone did not affect caspase-1 activation (Fig. 3A).

3.3. Lactobacillus S-layer proteins inhibit caspase-3 activation

To assess the effects on caspase-3 activation after infection of intestinal epithelial cells with *Salmonella* Typhimurium SL1344, we determined the time course of caspase-3 activation after infection. *Salmonella*-induced apoptosis was accompanied by changes in caspase-3 activity, as assessed by cleavage of the caspase-3 substrate Ac-DEVD-pNA. The caspase-3 activity initially decreased and then increased (Fig. 3B). There was a significant decrease in caspase-3 activity at 4 h (16.50 ± 2.74% reduction, $P = 0.009$) and 8 h (43.10 ± 3.23% reduction, $P < 0.001$) after infection with *Salmonella* Typhimurium SL1344 compared with control uninfected cells. In contrast, the caspase-3 activity was increased at 24 h (27.26 ± 1.63% increment, $P < 0.001$) and markedly reduced at 36 h (45.22 ± 2.91% reduction, $P < 0.001$).

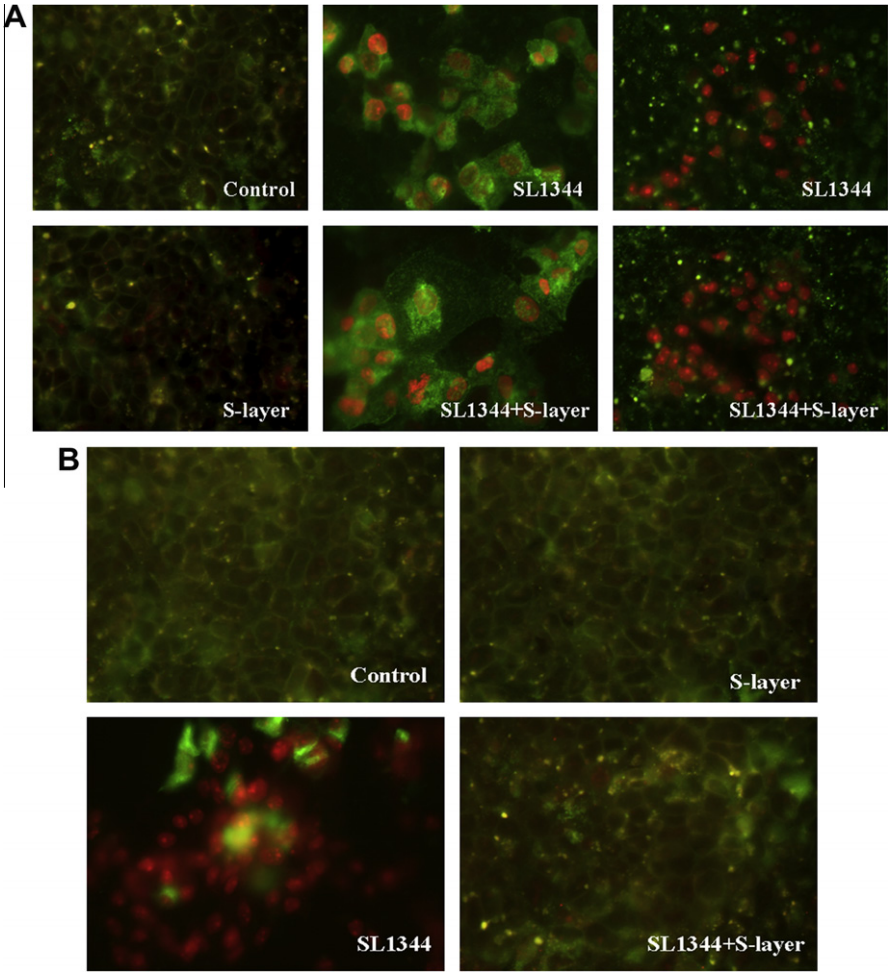

Fig. 2. *Lactobacillus* S-layer protein-mediated inhibition of *Salmonella*-induced apoptosis observed by microscopy (400×). (A) Caco-2 cells were coincubated with *Salmonella* Typhimurium SL1344 and *Lactobacillus* S-layer proteins for 24 h. Among Caco-2 cells infected with *Salmonella* Typhimurium SL1344 for 24 h, many apoptotic cells and necrotic cells show red and green fluorescence. Control cells and cells treated with S-layer proteins alone show little green fluorescence or no fluorescence, indicating few apoptotic cells. The S-layer proteins do not affect the change in *Salmonella*-induced apoptosis in Caco-2 cells at this time point. (B) Caco-2 cells were coincubated with *Salmonella* Typhimurium SL1344 and *Lactobacillus* S-layer proteins for 36 h. Among Caco-2 cells infected with *Salmonella* for 36 h, a large number of necrotic cells and some detached cells exhibiting strong red fluorescence are observed. However, S-layer proteins significantly inhibit the apoptosis-induced cell injury. The cells show little fluorescence and are restored to their former compact condition. The control cells received no treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As shown in Fig. 3B, there was a significant decrease in caspase-3 activity in Caco-2 cells in the presence of S-layer proteins compared with their absence at 4 h (11.35 ± 0.84% reduction). The caspase-3 activity was inhibited by S-layer proteins at 8 h (40.62 ± 0.44% reduction, $P < 0.001$), 24 h (35.32 ± 1.16% reduction, $P < 0.001$) and 36 h (21.62 ± 1.04% reduction, $P = 0.002$). When *Salmonella* Typhimurium SL1344 and S-layer proteins were coincubated simultaneously with Caco-2 monolayers, the caspase-3 activity showed no change at 4 h, was increased at 8 h and rapidly returned to the normal level at 24 h and 36 h.

3.4. Lactobacillus S-layer proteins activate the ERK1/2 signaling pathway

The reparation function in the intestinal mucosa, which maintains the barrier integrity of the epithelium, is mediated by ERK1/2 signal transduction [10]. Therefore, we investigated whether the ERK1/2 signaling pathway took part in the apoptotic process when Caco-2 cells were treated with *Salmonella* or S-layer proteins. The cells showed a significant increase in phospho-ERK1/2 activation after incubation with S-layer proteins alone (0.54 ± 0.04 relative band density) for 1 h compared with untreated Caco-2 cell monolayers (0.04 ± 0.01 relative band density) ($P = 0.001$; Fig. 4). We also found that *Salmonella* Typhimurium SL1344 activated ERK1/2 (0.34 ± 0.02 for infected cells versus 0.04 ± 0.01 for control uninfected cells, $P < 0.001$). Strikingly, when S-layer proteins and *Salmonella* Typhimurium were coincubated for 1 h with Caco-2 cells, the ERK1/2 phosphorylation (0.12 ± 0.01) was markedly suppressed compared with each treatment alone ($P < 0.001$).

4. Discussion

Different bacterial strains that cause cell apoptosis are selective for epithelial cell types [1,2,16]. In HT-29 human intestinal epithelial cells infected with *Salmonella* enterica serovar Dublin, apoptosis is preceded by activation of caspase-3, but not caspase-1 [1,2]. In contrast, *Salmonella* Typhimurium SL1344 activates caspase-1 in HeLa epithelial cells [16]. In the present study, *Salmonella* Typhimurium SL1344-induced apoptosis was analyzed in the Caco-2 human colon epithelial cell line. Caco-2 cells did not show activation of caspase-1 after infection with *Salmonella* Typhimurium SL1344, whereas caspase-3 was remarkably activated. These findings indicate that in Caco-2 cells infected with *Salmonella* Typhimurium SL1344, apoptosis is mediated by caspase-3 activa-

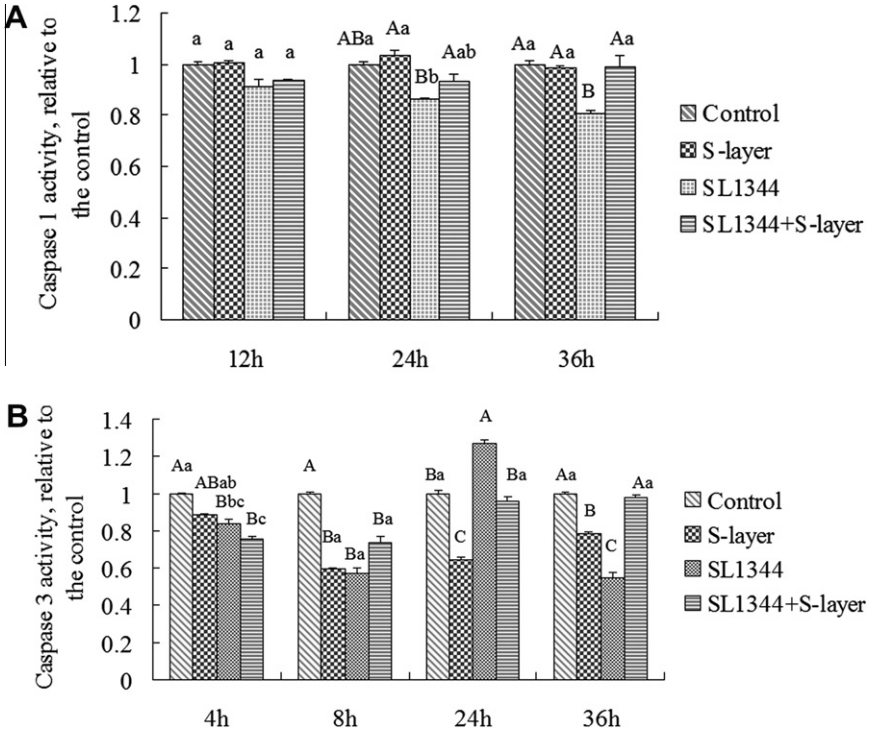

Fig. 3. Effects on caspase-1 and caspase-3 activities in Caco-2 cells after coincubation with S-layer proteins and *Salmonella* Typhimurium SL1344. (A) Caspase-1 activity assays. (B) Caspase-3 activity assays. The relative caspase-1 and caspase-3 activities were calculated as ratios of the cleavage of their substrates in treated cells relative to control cells, and the values of the control cells were set to 1. The data shown are the means ± SEM of three independent experiments for each protocol. The levels of significance are identified by letters as follows: bars with capital letters differ at $P < 0.01$; bars with small letters differ at $P < 0.05$. The control cells received no treatment.

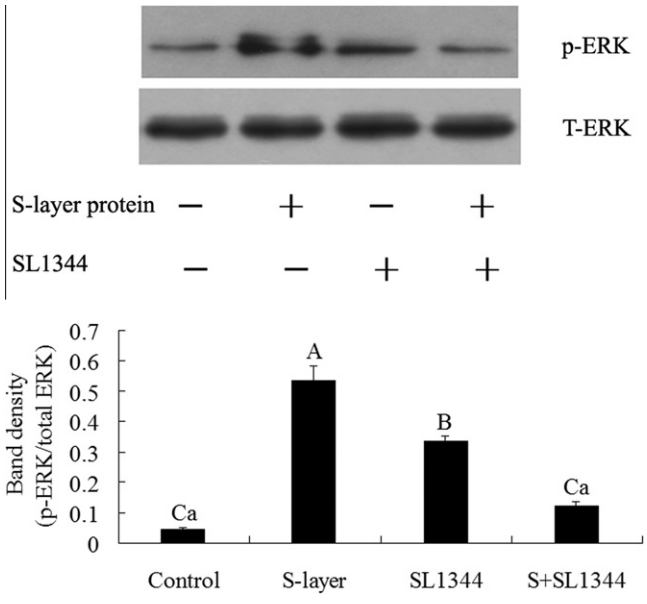

Fig. 4. *Lactobacillus* S-layer proteins can activate the ERK1/2 signaling pathway in Caco-2 cells. The ERK1/2 phosphorylation level (p-ERK) was assessed by Western blotting analysis. To confirm equal loading, the membranes were stripped and reprobed for total ERK1/2 (T-ERK). The data shown are the means ± SEM of triplicate wells from three independent experiments. The levels of significance are identified by letters as follows: bars with capital letters differ at $P < 0.01$; bars with small letters differ at $P < 0.05$. The control cells received no treatment.

tion. The activation of certain caspases in different epithelial cells after *Salmonella* infection suggests that these cells may undergo apoptosis through multiple pathways. Although we found that caspase-1 activity was reduced at 24 h and 36 h post-infection with *Salmonella* Typhimurium SL1344 in Caco-2 cells, this may have been associated with cell damage caused by *Salmonella*-induced apoptosis. In our study, caspase-3 activity in Caco-2 cells was first downregulated at 8 h and during the following time period after infection with *Salmonella* Typhimurium SL1344. Our findings suggest that the delay in onset of *Salmonella*-induced apoptosis may be executed by inhibition of caspase-3 activation.

In the present study, we found that the inhibition of *Salmonella*-induced apoptosis was mediated by *Lactobacillus* S-layer proteins in Caco-2 cells. The underlying mechanism may involve the pathway of S-layer protein-inhibited caspase-3 activation. We found that the caspase-3 activity was decreased at 8 h after infection with *Salmonella*, while S-layer proteins could also inhibit the activity of caspase-3. Interestingly, when Caco-2 cells were treated with *Salmonella* and S-layer proteins simultaneously for 8 h, the caspase-3 activity was increased compared to cells treated with *Salmonella* or S-layer alone. The results implied that this might be related to a reduction in the number of invading bacteria by the S-layer proteins [14]. When *Salmonella* and S-layer proteins were coincubated for 24 h and 36 h, the S-layer proteins could antagonize the upregulation of caspase-3 activity caused by *Salmonella* infection. This antagonistic activity of the S-layer proteins may be directly involved in their inhibition of caspase-3 activation. In Caco-2 cells after infection with *Salmonella* alone for 36 h, a large number of apoptotic and necrotic cells were observed and some cells had become detached. This phenomenon was correspond with the caspase-3 activity was markedly reduced at 36 h.

However, S-layer proteins significantly inhibited the apoptotic cell injury at 36 h after infection with *Salmonella*. Microscopic observations also showed that the apoptotic cells were significantly reduced, and the cells were restored to their former compact condition. These findings suggest that the antagonistic activity of S-layer proteins also includes a modification function for the apoptotic cell damage caused by pathogens. We speculate that the main reason behind this process is that some of the infected epithelial

cells harboring pathogens could ultimately be eliminated by apoptosis. Furthermore, the gentamicin in the tissue culture medium would kill any extracellular bacteria released from the apoptotic cells, but have no effect on the intracellular bacteria [17]. This would prevent the extracellular bacteria from causing another infection, and would also be of benefit for restoring the normal regulation of uninfected epithelial cell growth and differentiation, while preserving the integrity of the epithelial barrier. This process also offers an explanation for why caspase-1 activity was restored to the normal level when *Salmonella* and S-layer proteins were coincubated simultaneously for 36 h.

We found that the S-layer proteins mediated marked ERK1/2 activation in Caco-2 cells, consistent with the results for the inhibition of caspase-3 activity by these proteins. ERK1/2 signal transduction mainly mediates primary proliferation and differentiation [18]. Therefore, the findings suggest that *Salmonella*-induced apoptotic cell damage may be modified by S-layer proteins through the ERK1/2 signaling pathway. In the initial stage of *Salmonella* infection, the ERK1/2 signaling pathway was activated. The delayed *Salmonella*-induced apoptosis may also be executed through the ERK1/2 signaling pathway by inducing cell proliferation and differentiation. Strikingly, S-layer proteins were able to suppress *Salmonella*-induced ERK1/2 phosphorylation. S-layer proteins have the ability to autoassemble on bacterial surfaces [13,19]. S-layer proteins may be associated with the *Salmonella* surface, and could interact with specific sites on the bacterial surface involved in the first step of mucosal infection. Alternatively, S-layer proteins could either modify or mask the *Salmonella* structures necessary for secreting the effectors of the ERK1/2 signaling pathway to inhibit its activation.

In conclusion, the present results suggest that in Caco-2 cells infected with *Salmonella* Typhimurium SL1344, apoptosis is mediated by caspase-3 activation. We have demonstrated that the antagonistic activity of S-layer proteins may be directly involved in the process by reducing downstream caspase-3 activation, and that modification of the apoptotic cell damage is linked to activation of the ERK1/2 signaling pathway. On the basis of these encouraging in vitro results, further investigations should be carried out to characterize the apoptotic inhibitory effects and the in vivo activity of *Lactobacillus* S-layer proteins against pathogenic bacterial infections in host animals.

Conflicts of interest

None.

Acknowledgments

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