Scientific Research Publishing

Advances in Microbiology, 2016, 6, 942-952
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Survival, Proliferation and Cell Cycle of Swine Fibroblast after Infection with *Salmonella enterica*

Mizuki Masuda[1], Yijie Guo[1,2], Kengo Kuroda[1], Jun Xu[1], Hiroshi Yoneyama[1], Tomokazu Fukuda[3], Bernard Mudenda Hang'ombe[4], Katsuya Okuno[1], Junko Nishimura[5*], Emiko Isogai[1*]

[1]Laboratory of Animal Microbiology, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan
[2]Department of Immunobiology and Pathogenic Biology, Medical School of Xi'an Jiaotong University, Xi'an, China
[3]Laboratory of Cell Engineering and Molecular Genetics, Faculty of Science and Engineering, Iwate University, Morioka, Japan
[4]Department of Clinical Microbiology, School of Veterinary Medicine, University of Zambia, Lusaka, Zambia
[5]Laboratory of Nutrition and Life Sciences, Department of Biotechnology and Environmental Engineering, Faculty of Engineering, Hachinohe Institute of Technology, Hachinohe, Japan
Email: *emiko@bios.tohoku.ac.jp, *jnishimura@hi-tech.ac.jp

How to cite this paper: Masuda, M., Guo, Y.J., Kuroda, K., Xu, J., Yoneyama, H., Fukuda, T., Hang'ombe, B.M., Okuno, K., Nishimura, J. and Isogai, E. (2016) Survival, Proliferation and Cell Cycle of Swine Fibroblast after Infection with *Salmonella enterica*. *Advances in Microbiology*, **6**, 942-952.
http://dx.doi.org/10.4236/aim.2016.613088

Received: August 11, 2016
Accepted: November 6, 2016
Published: November 9, 2016

Abstract

Food-borne salmonellosis continues to be a major health concern worldwide. Carry-contamination of *Salmonella* frequently occurs in meat production. We focused on cell dynamics of swine fibroblasts after infection with *Salmonella enterica* serovar Enteritidis and Typhimurium, because fibroblast can be a target cell for *Salmonella* latent infection. It was found that both *S.* Enteritidis and *S.* Typhimurium were able to adhere and invade to swine fibroblasts. The proliferations in fibroblasts were different between each serovar. *S.* Enteritidis reached to the maximum at 24 hr after infection while *S.* Typhimurium did not. In addition, the decrease in the G_0/G_1 phase cells and increase in G_2/M phase cells on the fibroblast were observed by both *Salmonella* infection. Cell death including apoptosis in the cells was inhibited by the infection of *Salmonella*. These results suggest that nontyphoidal *Salmonella* can survive for the long term by modifying bacterial cell proliferation and preventing cell death of host cells.

Keywords

Salmonella enterica, Fibroblast, Swine, Cell Death, Cell Cycle

1. Introduction

Salmonella enterica causes various diseases, particularly, nontyphoidal salmonellae are very important in reportable food-borne infections. *Salmonella* is an intracellular pathogen that can invade eukaryotic cells and manage to survive in the living host cell [1]

[2] [3]. It is well known that this bacteria is able to invade via Type Three Secretion System (TTSS) encoded in *Salmonella* Pathogenicity Island 1 (SPI-1) [4]. When *Salmonella* invades to host cells, the SPI-1 effector protein of the TTSS is injected into epithelial cells, thereby causing rearrangement of actin cytoskeleton [5] [6] [7], membrane ruffling and formation of micropinosomes [8]. As a case of cytoskeletal rearrangement, it is revealed that SPI-1 proteins SipABCD, SopE and SopE2 are involved [9].

In the present, it has been found that the infection route of *Salmonella* Typhimurium against fibroblast cells was quite different from that of epithelial cells, in which the SPI-1 effectors SipB and SipC were unnecessary. And this strain is able to suppress cell growth by stopping cell division in fibroblast cells after invasion [10] [11] [12]. Based on these findings, it is considered that *S.* Typhimurium could alter the routes into fibroblasts, and persistent infection and asymptomatic carrier are caused.

Although it has been known the importance of salmonellosis to public health, the mechanism of the *Salmonella* carrier state hasn't been well known still now. We examined the invasion of *Salmonella* into fibroblasts, because there was a risk of giving rise to the persistent infection. In addition, we show here invasion and proliferation of *S.* Typhimurium in the fibroblasts differs from that of *S.* Enteritidis.

2. Materials and Methods

2.1. Bacterial Strains and Swine Fibroblast Cell Line

Salmonella enterica serovar Enteritidis strain zSE1 isolated in Zambia and Typhimurium wild type strain st1wt were cultured properly in Trypticase Soy Broth (TSB) at 37˚C for 18 hr [13]. Pig embryonic fibroblasts (PEFs), which infected with simian vacuolating virus 40 large T fragment (PEFs-SV40) to achieve immortalization, used in this study [14]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque Inc., Kyoto, Japan) with 10% PBS and 1% antibiotic-antimycotic mixed stock solution (Nacalai Tesque Inc.) at 37˚C in 5% CO_2. Cells were maintained under exponential growth condition and used as the host cells in further experiments.

2.2. Bacterial Infection Assays

PEFs were seeded in a 24-well-plate to reach a density of 1.0×10^5 cells/well at the time of infection. The medium was changed to DMEM with 10% FBS (without antibiotics) 2 hr before bacterial infection to eliminate any potential effects of the antibiotics. PEFs were infected with overnight cultured bacteria at a multiplicity of infection (MOI) of 5:1 (bacteria to eukaryotic cells). To count the adhesive bacteria at 0, 20, 60, and 100 min after the infection, wells were washed with PBS containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 1% (vol/vol) Triton X-100 (Wako Pure Chemical Industries Ltd.) as lysis buffer. The invasive bacteria were counted, and cells were washed with PBS repeatedly. Fresh culture medium containing 100 μg/ml gentamicin was added for 2 hr post-infection, and then the cultured cells were lysed with lysis buffer.

2.3. Immunofluorescence Microscopy

Extracellular (adherent) and intracellular bacteria were stained by immunofluorescent microscopy by the methods of Aiastui *et al*. [10]. Briefly, extracellular bacteria were stained in nonpermeabilized cells with polyclonal rabbit anti-*Salmonella* lipopolysaccharide (LPS) antibodies (*S.* Enteritidis O4 and *S.* Typhimurium O9, Denka Seiken Co., Ltd, Tokyo, Japan), followed by anti-rabbit Alexa Fluor 594 F(ab')2 fragment antibody (Invitrogen, CA, USA). Upon permiabilization by treatment with 0.2% Triton X-100, intracellular bacteria were stained with anti-Salmonella LPS antibody above, followed by anti-rabbit Alxa-Fluor 488 F(ab')2 fragment antibody (Invitrogen) as secondary antibodies.Cells were observed with a Fluorescent microscope (FSX 100, Olympus, Tokyo, Japan).

2.4. Intracellular Proliferation

Fresh DMEM medium containing 100 μg/ml gentamicin was added into 2 hr infected fibroblast. After cultivation for 24 hr, PEFs were dissolved in lysis buffer and intracellular bacterial numbers were counted.

2.5. Live Cell Count

Bacteria-infected PEFs (MOI = 5:1 as described above) were treated at 0, 20, 60, 100 min after the infection with 0.05% Triton X-100 and cells were collected. Cells were mixed with trypan blue (Invitrogen) and living cells were counted using a CSTI Counter (Cell Science & Technology Institute, Inc., Miyagi, Japan). In order to assess the effects of intracellular pathogens and long-term infection, the medium was changed to fresh medium containing 100 μg/ml gentamicin 2 hr after infection. After gentamicin treatment for 24 hr, live PEFs were counted according to mentioned.

2.6. MTT Assay

PEFs were seeded in a 96-well-plate and incubated for 24 hr to reach a density of 3.0×10^3 cells/well at the time of infection. The medium was changed to DMEM with 10% FBS (without antibiotics) 2 hr before bacterial infection to eliminate any antibiotic effects. Cells were infected with *Salmonella* (MOI = 5:1), and then 10 μl of MTT reagent from the MTT Cell Proliferation Assay kit (Funakoshi Co., Ltd., Tokyo, Japan) was added immediately after infection. After post-infection for 2 hr, the medium was changed to DMEM containing 100 μg/ml gentamicin, and MTT reagent was added to each samples at 0, 2, and 24 hr.

2.7. Apoptosis and Cell Cycle Assay

Apoptosis of infected cell was analyzed with Muse™ Cell Analyzer (Merck Millipore Inc., Darmstadt, Germany).PEFs were inoculated 0.1×10^5 cells per well. *S.* Enteritidis or *S.* Typhimurium was added into well in the ratio of 5:1 (MOI) and incubated for 2 hr. After infection, PEFs were washed with PBS and collected with 0.05% trypsin. And then PEFs were centrifuged (800 × g) and washed with PBS. Infected PEFs were col-

lected with 0.05% trypsin and added Muse Annexin & Dead Cell Reagent and standing for 30 min at room temperature in a dark place. Apoptotic cells were detected with the analyzer. Furthermore, apoptosis profile of PEFs infected with *S.* Enteritidis zSE1 after infection for 24 hr was monitored using the flow cytometry.

The cell cycle of PEFs was also analyzed by using the Muse™ Cell Cycle Assay Kit (Merck Millipore Inc.), according to the procedure described by the manufacturer. Infected PEFs were collected with 0.05% trypsin, centrifuged (800 × g), and washed with PBS. Collected cells were suspended in cold 70% ethanol and incubated for over 3 hr at −20˚C for fixation. Fixed cells were washed with PBS and stained with the cell cycle reagent for 30 min. Samples were measured by using the above analyzer.

2.8. Statistical Test

In this study, all of the experiments were carried out with at least triplicated samples. Mean and standard deviations were calculated from the multiple data. The statistical significance was evaluated unpaired *t*-test. After statistical analysis, *p* values of less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Infection to Swine Fibroblasts

Although it has been revealed that fibroblasts are highly involved in the persistence of pathogenic *Salmonella* [15], the mechanisms of long-term and persistent infection of *Salmonella enterica* are still unknown. In this study, we investigated *Salmonella*-fibroblast interactions to clarify the survival strategy of *Salmonella* in the host fibroblasts.

We first confirmed the infection of *Salmonella* to PEFs by conducting infection assay and immunofluorescence. Each cell of *S.* Enteritidis and Typhimurium adhered approximately 1% for a start, and adherent cell number was increased time-dependently (**Figure 1(a)**). At 100 min, adherent number of *S.* Typhimurium was significantly higher than that of *S.* Enteritidis ($P < 0.01$). Cell invasion was observed after 60 min of the infection. *S.* Typhimurium invaded cells more aggressively than *S.* Enteritidis (**Figure 1(b)**, $P < 0.01$ at 60 min after the infection). The states infected with *S.* Enteritidis or *S.* Typhimurium were photographed with a fluorescent microscope and typical photo-images at 24 hr after *S.* Typhimurium infection were showed (**Figure 2**). Furthermore, the intracellular proliferation of *Salmonella* cells was different between *S.* Enteritidis and *S.* Typhimurium, *i.e.*, *S.* Enteritidis reproduced sharply after invasion to PEFs, on the other hand, *S.* Typhimurium was almost not (**Figure 3**, $P < 0.01$). Namely, *S.* Enteritidis zSE1 reached 1.28×10^4 cfu after infection for 24 hr, while *S.* Typhimurium st1wt couldn't proliferate in host cells and only reached 3.3×10^2 cfu.

3.2. Influences to Viability and Lifespan of Host Fibroblast

In order to assess the influence of host cells infected with pathogenic *Salmonella,* we analyzed the viability and proliferation of infected PEFs. The number of living infected

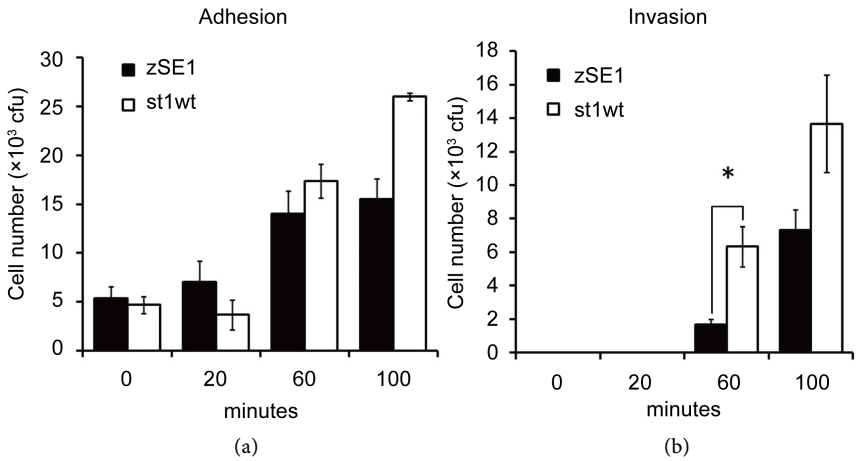


Figure 1. Chronological changes of adhesion and invasion on *Salmonella enterica*. (a) Cell numbers of *S.* Enteritidis or Typhimurium which can adhere to PEFs. (b) Cell numbers of *S.* Enteritidis or Typhimurium which can invade to PEFs. *: P<0.01 unpaired *t*-test.

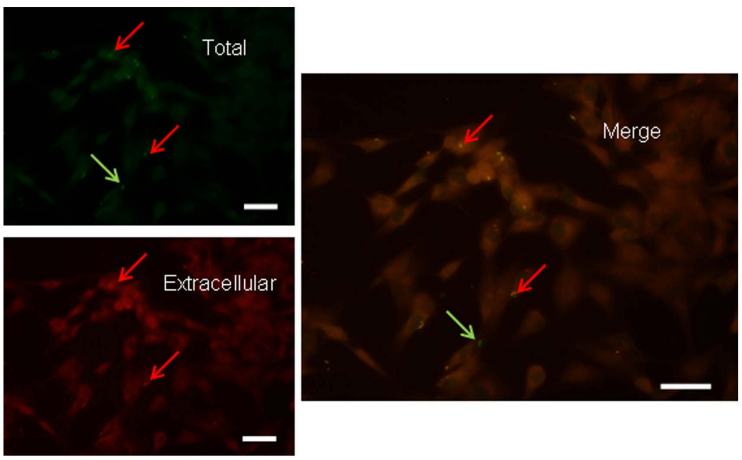


Figure 2. Fluorescent microscopy photographs of *S.* Typhimurium after infection for 24 hr. Bar: 50 μm. Red arrow: adhesion, green arrow: invasion.

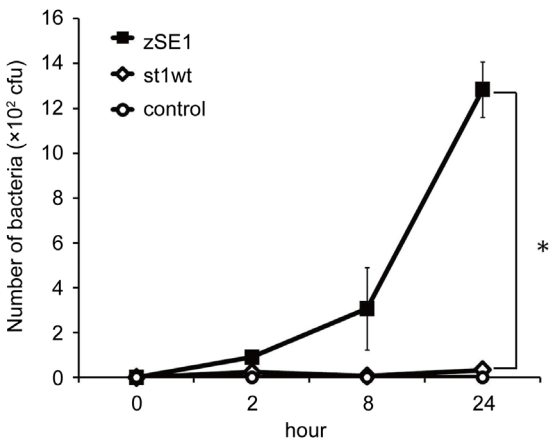


Figure 3. Chronological changes of bacterial numbers on intracellular *Salmonella enterica*. *: P < 0.01, unpaired *t*-test.

PEFs was counted after trypan blue staining. As a result, it was found that living cells were increased after infection with both *Salmonella* strains compared to non-infected cells (Figure 4). The proliferation of living PEFs was measured by MTT assay after 0, 2 and 24 hr infection (Figure 5). The proliferation of PEFs infected for 24 hr was remarkably increased, particularly, a significant difference was revealed on *S.* Typhimurium. These results indicate that *Salmonella* is able to survive within host fibroblasts and enhance the proliferation of host cells. It is found that *S.* Typhimurium led to enhance the proliferation in epithelial cells [16] and suppress it in dendritic cells and fibroblasts [11] [17]. Furthermore, *S.* Enteritidis into human fibroblasts increased during 1 day after infection and could survive until 14 to 28 days [18]. Based on these findings, it is considered that *Salmonella* grows rapidly in fibroblast and gradually controls

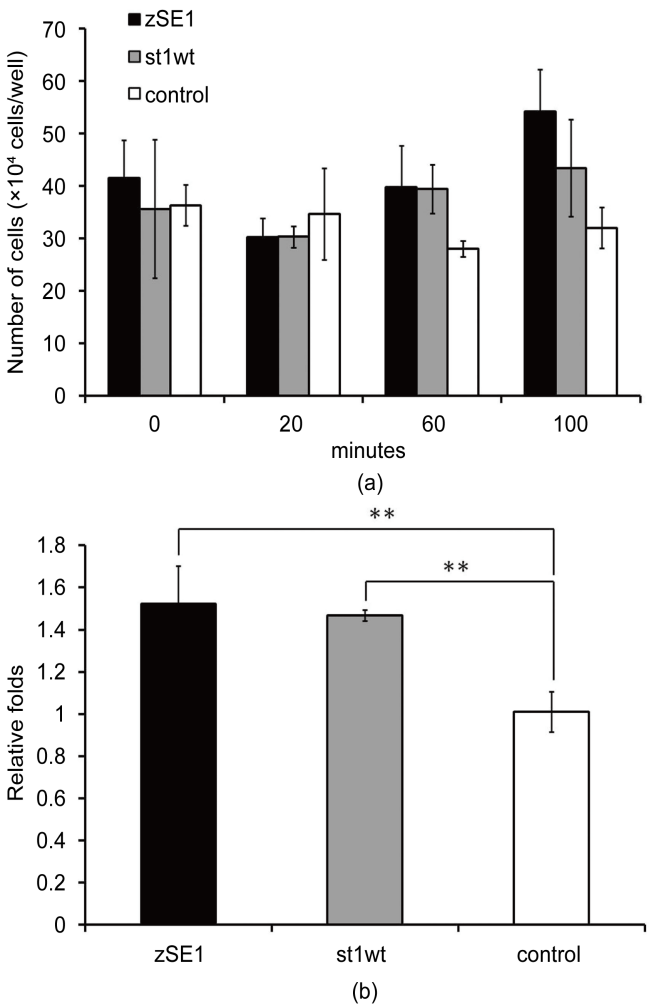


Figure 4. Proliferation of PEFs infected with zSE1 or st1wt. (a) The number of PEFs infected with *S.* Enteritidis or Typhimurium and non-infected-PEFs were counted using the trypan blue method. 0, 20, 60, 100 min after infection, cell number of PEFs infected with bacteria tended to be larger that of non-infected PEFs. (b) 24 hr after infection, PEFs infected with zSE1 or st1wt were about 1.52 fold or 1.47 fold respectively compared with non-infected PEFs. **: $P < 0.05$, unpaired *t*-test.

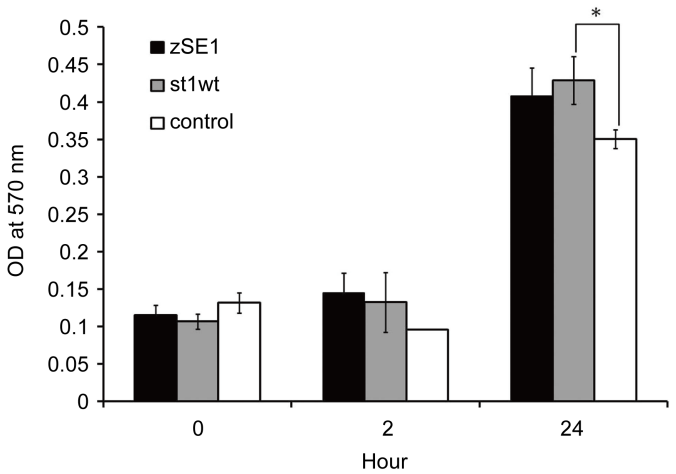


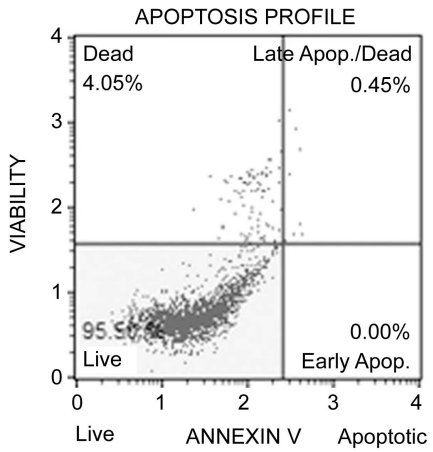
Figure 5. Time-lapse changes of cell proliferation on host PEFs. Cell viability was measured by MTT cell proliferation assay. Colorimetrical formazan produced from live cell metabolism were detected as absorbance at 570 nm. *: $P < 0.01$ unpaired *t*-test.

them after that, and *Salmonella* is possible long-term survival by repressing the proliferation in restricted host cells.

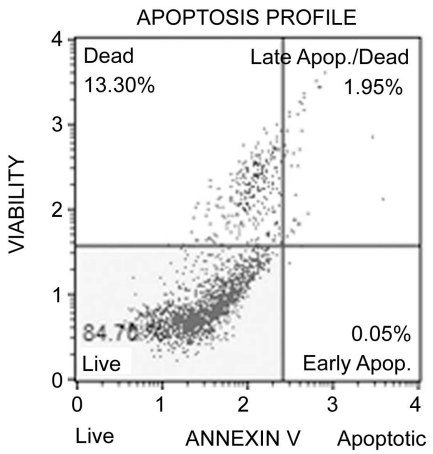
3.3. Effect to Viability of Fibroblast

We demonstrated the cell death pattern of host cells after *Salmonella* infection as shown in **Figure 6**. As shown in **Figure 7**, apoptotic cells counted was 1% - 1.5% (significantly higher than control) at the beginning of the infection. After 24 hr infection, the percentage of apoptotic cells significantly increased in non-infected cells; in contrast, the percentage of apoptotic cells was approximately 0.5% on *Salmonella* infected cells. Total dead cell number was also decreased by the *Salmonella* infection (data not shown).

To further explain such phenomenon, cell cycle of infected cells was analyzed by using a Cell Analyzer. The cell cycle of infected or non-infected PEFs was measured by flow cytometry 24 hr after addition of bacteria. The results revealed that G_0/G_1 phases of infected cells were reduced and the G_2/M phases were prolonged compared with non-infected cells, respectively (**Figure 8**). It has been found that enteropathogenic *Escherichia coli* and enterohaemorrhagic *E. coli* infused effector protein Cif to eukaryotic host cells and led to arrest cell cycle, and the barrier of the epithelium cell became weak by stopping a cell cycle, and bacteria became easy to infect cells [19]. And the G_0 phase was longer to repair DNA in cell cycle [20], the apoptosis was caused when DNA damage could not repair [21] [22]. In addition, it has been known that *Salmonella* led to induce apoptosis of host chicken fibroblasts [23], and the ratio of apoptotic PEFs infected with *Salmonella* Enteritidis or Typhimurium might be increased. Based on these findings, it seems that *Salmonella* infection induces the alteration of the cell cycle on host PEFs, and interrupts apoptosis of the host cell to survive in host cells. Namely, *Salmonella* of the intracellular parasitism may lead to repress cell cycle after invasion.



(a)



(b)

Figure 6. Dead /live cells and apoptosis profile of PEFs infected with *S.* Enteritidis zSE1. (a) Infected cells. (b) Control: non-infected cells.

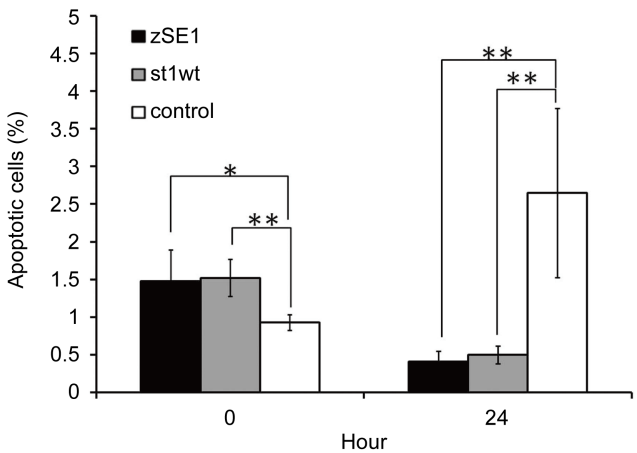


Figure 7. Proportion of apoptotic PEFs after infection with *Salmonella enterica*. Apoptosis caused by *Salmonella enterica* infection were detected by Annexin V using flow cytometry. *: $P < 0.01$ and **$P < 0.05$, unpaired *t*-test.

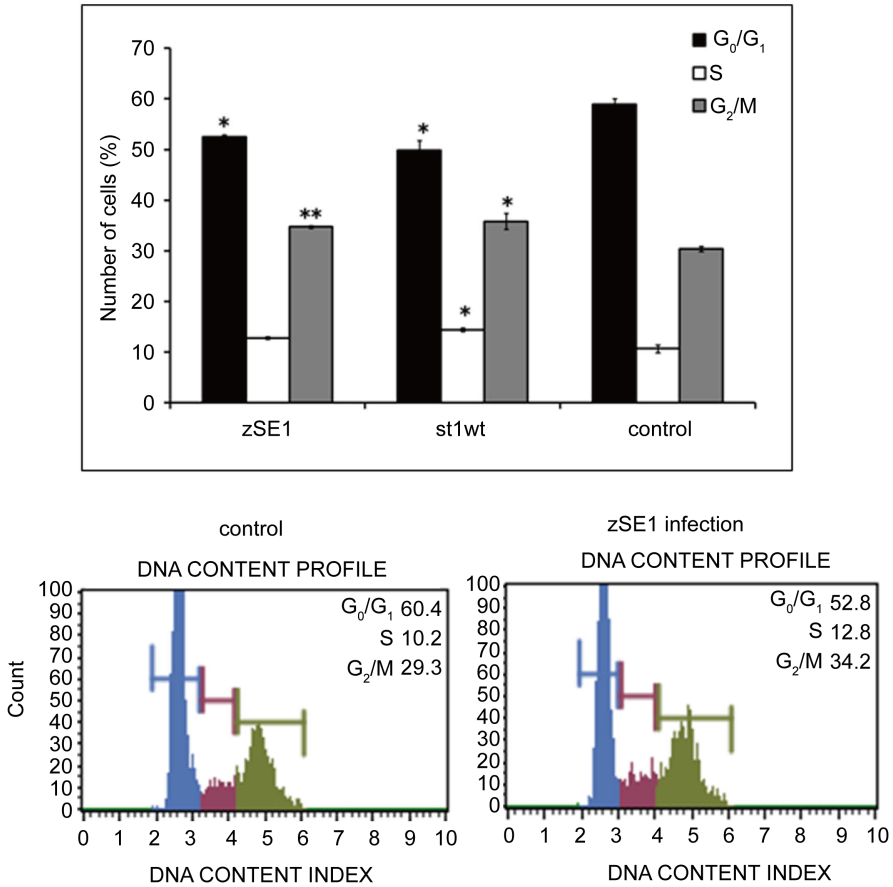


Figure 8. The ratio of cell cycle phase in PEFs after infection with *Salmonella enterica*. *: $P < 0.01$ and **: $P < 0.05$, unpaired *t*-test.

It has been known that bacteria are able to suit host cell environment by promoting the most successful conditions for infection [24]. In this study, we demonstrated that *Salmonella* was able to survive in fibroblasts by manipulating both lifespan and apoptosis of host cell. This phenomenon is intended to optimize the circumstances of *Salmonella* survival and promote the persistent infection of *Salmonella* in livestock. This series of strategy could be a notable fact that highlights new concepts of *Salmonella* infection of fibroblasts in domestic animals, and encourages people to reconsider the hidden issue in food safety.

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