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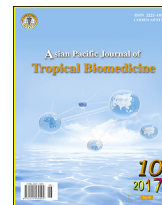

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Comparing invasive effects of five foodborne bacterial pathogens in human embryonic intestine 407 cells and human ileocecum HCT-8 cells

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ABSTRACT

Objective: To refine the infectious doses of enteric bacterial pathogens in animal assays and vaccine clinical trials by studying the invasion kinetics of five bacterial pathogens with human intestinal cells.

Methods: Utilizing *in vitro* cultured cell invasion assays with gentamicin-killing step, the invasive effects were analyzed in foodborne pathogens including *Salmonella*, *Shigella, Yersinia*, *Escherichia coli* (*E. coli*) O157 and opportunistic pathogens *Citrobacter* in human embryonic intestine 407 cells and ileocecum HCT-8 cells at multiplicities of infection (MOIs) of 0.04–4 000.00 *E. coli* HS served as a noninvasive control.

Results: The study results showed that the bacterial invasive efficiency and the average number of internalized bacteria per host cell changed with different starting MOIs. Higher starting MOIs did not always produce more bacterial internalization. The bacterial invasion effects varied with different bacterial strains and host cell lines. *E. coli* O157:H7 did invade human ileocecum HCT-8 cells.

Conclusions: This study shows that these bacteria possess different invasive patterns at various starting MOIs and also in different cell lines. The results could help to figure out the appropriate infectious doses of the bacteria in animal assays and in vaccine clinical trials. The bacterial invasion kinetics is also valuable in evaluating the safety and efficacy of live attenuated bacterial vaccines.

1. Introduction

Enteric pathogenic bacteria such as *Salmonella*, *Shigella*, *Yersinia*, and Shiga toxin-producing enterohemorrhagic *Escherichia coli* (*E. coli*) (EHEC) O157:H7 are the most common foodborne bacterial pathogens. These bacteria cause human gastroenteritis, diarrhea, septicemia and other systemic diseases. *Citrobacter* species are gram-negative bacteria, and can also colonize the intestinal tracts of humans and animals. They are opportunistic pathogens and associated with a wide spectrum of infections involving the gastrointestinal, urinary, and respiratory tracts, as well as central nervous system of humans [1]. Foodborne diseases are a critical public health concern. The number of reported cases of foodborne disease per year in the US is approximate 9.4 billion with 55 961 hospitalizations and 1 351 deaths [2]. Foodborne diseases are estimated to cause more than 2 million deaths per year worldwide [3]. *Shigella* species cause bacillary dysentery with an estimated 90 million cases and 100 000 deaths per year in the developing world, with an especially high incidence (about 60%) among children younger than 5 years of age [4,5]. Typhoid fever, a systemic infection caused by *Salmonella enterica* serotype *Salmonella typhi* (*S. typhi*) and *Salmonella paratyphi* remains an important public health problem in less developed countries. It is estimated that over 27 million cases of typhoid occur worldwide annually with approximate 217 000 deaths, most of the cases occurring in Asia and Africa [6,7].

To establish and maintain a successful infection, bacterial pathogens have evolved a variety of strategies including invasion and multiplication within their host, avoidance or resistance to human innate immune response, or damage of the gastrointestinal system. The first critical step for causing disease by invasive bacteria is their ability to subvert host factors to induce their uptake into normally nonphagocytic epithelial cells [8–10]. The infectious dose to cause an infection varies with organisms. Some intestinal pathogens can start an infection

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with only a small number of cells. For example, as few as 10 *Shigella* cells are sufficient to cause an infection resulting in mucosal ulceration and bloody diarrhea [11]. EHEC are also highly infectious and have a low infectious dose, with as few as 10 bacterial cells being sufficient to cause disease [12]. And 100–1 000 *Salmonella* cells can cause infection. Some pathogens are only able to cause infections at higher infectious doses. For example, *Yersinia enterocolitis* causes an infection with 10^{6-9} bacterial cells [13].

After ingestion *via* contaminated food or water, bacteria have to pass through the highly acid gastric fluids in the stomach and the strongly alkaline secretions from the bile duct in the upper small intestine. The infectious dose of bacteria may be affected by gastrointestinal pH and host sensitivity. Reduced stomach acidity by antacid agents may reduce the number of bacteria needed to cause an infection. Also, certain foods or milk products protect the organisms from being killed by gastric fluids. Pathogens with low infectious doses may be easily transmitted by food handlers. Those with higher infectious doses are less likely to cause diseases, but improper food processing, handling and storage conditions can also lead to an increase in these bacterial infections. Moreover, the invasive ability of bacteria to internalize into host cells is an important factor for affecting the infectious dose.

Infection of cultured intestinal epithelial cells by bacteria has extensively been applied to study the interaction of enteric bacteria with host cells. Although many bacteria have been reported to invade into intestinal epithelial cells, the invasive efficiency, average number of internalized bacteria per host cell and the detrimental effects of enteric bacteria to host cells have not been well studied in human intestinal cells using various multiplicities of infections (MOIs). Therefore, this study was conducted to compare the invasion effects of foodborne bacteria including *Salmonella*, *Shigella*, *Yersinia, E. coli* O157, and *Citrobacter* at MOIs of 0.04–4 000 in human embryonic intestine INT407 and human ileocecum HCT-8 cells. Understanding the invasion kinetics of the enteric pathogenic bacteria with human intestinal cells will help to refine the infectious doses of these bacteria in animal assays and vaccine clinical trials. The knowledge is also valuable in evaluating the safety and efficacy of live attenuated bacterial vaccines.

2. Materials and methods

2.1. Bacterial strains, cell lines, media and culture conditions

S. typhi Ty2, *Shigella flexneri* (*S. flexneri*) 2a M4243 (wild type, Sul^r, including large plasmids and a small cryptic plasmid), *E. coli* O157:H7 C8632, and *Citrobacter freundii (C. freundii)* 7004 are all wild type strains. These strains were obtained from Walter Reed Army Institute of Research except *E. coli* O157 C8632 which was isolated from a patient with hemolytic uremic syndrome (American Type Culture Collection). *E. coli* #375 (Amp^r, Cm^r, *inv*$^+$) kindly provided by R. Isberg (Tufts University) contains a functional *Yersinia* invasin gene *inv*$^+$, which is cloned into a plasmid pRI203 in *E. coli* HB101. *E. coli* HS (Walter Reed Army Institute of Research) serves as a noninvasive control.

These bacteria were cultured in Luria–Bertani (LB) broth, and frozen stocks were maintained in LB with 10% dimethyl sulfoxide at −80 °C. Human embryonic intestine (INT) 407 cells and human ileocecum HCT-8 cells were obtained from the American Type Culture Collection. The INT407 cells were cultivated in minimal essential media (MEM) with 10% heat-inactivated fetal bovine serum (Invitrogen), 0.2 mM L-glutamine and 0.1 mM nonessential amino acids. The HCT-8 cells were cultured in RPMI1640 with 10% fetal calf serum, 1 mM sodium pyruvate, 0.2 mM L-glutamine and 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid.

2.2. Invasion assays

These assays were performed as described previously [14]. The human INT407 or HCT-8 cells at a concentration of 10^5/well were added to a 24-well cell culture plate and were incubated for 24 h. Mid-log phase bacteria in MEM were added to 1 mL of culture media per well. The MOIs varied in the assays as indicated. The infected monolayers were typically incubated for 2 h at 37 °C at 5% CO_2 and 95% air atmosphere to allow invasion to occur. Following this invasion period, the monolayers were washed three times with MEM, and then incubated for another 2 h in fresh tissue culture medium containing 100 µg/mL gentamicin to kill extracellular bacteria. After the gentamicin killing period, the infected monolayers were washed three times, and the cells were lysed using 0.1% Triton X-100 in phosphate buffer saline for 15 min at room temperature on an orbital shaker. Following serial dilution in phosphate buffer saline, released intracellular bacteria were enumerated by colony count on LB agar plates at 37 °C. Each invasion assay was performed simultaneously in two separate wells, and was repeated at least 3 separate occasions. Results are presented as the mean ± SD. Experiments with controls confirmed that 100 µg/mL gentamicin killed essentially all extracellular bacteria (*i.e.*, > 99.99%) within 2 h.

2.3. Cytotoxicity assay

The CytoTox 96 assay (Promega, Madison, WI) was used to determine host cytotoxicity [15]. The assay qualitatively measures supernatant lactate dehydrogenase (LDH), and a stable cytosolic enzyme is released upon cell lysis. Maximum LDH release was determined by measuring the amount of LDH release from uninfected cells that were treated with lysis buffer. The percentage of cytotoxicity was calculated according to the following formula (OD is an abbreviation for optical density): $[(OD_{sample}) - (OD_{medium}/OD_{maxLDH\ release}) - OD_{adjusted\ medium}] \times 100$.

2.4. Statistical analysis

Results are presented as the mean ± SD from three independent assays. The means of the invasion assays were compared using Student's *t*-test.

3. Results

3.1. Invasion efficiency of foodborne pathogens internalized into human INT407 cells

To monitor the effects of the uptake of intestinal bacterial pathogens, human INT 407 cells were infected with *S. typhi* Ty2, *S. flexneri* 2a strain M4243, *E. coli* 375 (*inv*$^+$) containing

functional *inv* gene of *Yersinia*, *E. coli* O157:H7 strain C8632, *C. freundii* 7004 and noninvasive control *E. coli* HS. The invasion efficiency (*i.e.* percentage of the starting inoculum internalized at the end of the assay) of these bacteria were assessed over a wide range of starting bacterial concentrations (expressed as MOIs), from approximately 0.04 to 4 000 bacteria per INT407 cell in the gentamicin-killing assay. The resulting invasion efficiency of *S. typhi* Ty2 increased steadily from starting MOIs of approximately 0.04–40.00, where it reached a maximum of approximately 17% and decreased sharply and consistently thereafter (Figure 1A). The invasion efficiency was markedly lower at MOIs of 400 and 4 000 than at MOIs of approximately 4 and 40. *S. typhi* Ty2 showed an invasion optimum at an MOI of 40, possibly indicating that a natural infection required a higher dose of the organisms.

Similarly, *S. flexneri* M4243 increased from a starting MOI of approximately 0.04–0.40, where the invasion efficiency reached a maximum of approximately 2.5% and decreased gradually thereafter. The highest invasion efficiency of the organism occurred at an MOI of 0.4 (Figure 1B). This pattern is the same as the low infectious dose of *Shigella* (approximately 10 bacteria), which have been shown to cause disease in volunteer studies. In contrast, the invasion efficiency of *E. coli* #375 (inv^+) containing the *Yersinia* invasin gene maintained the same from MOI of 0.04 to MOI of 40, then decreased at higher MOIs (Figure 1C). The highest invasion efficiency occurred at the lowest MOI (0.04). We used *E. coli* #375 (inv^+) instead of a *Yersinia* strain to determine the invasion efficiency of *Yersinia* due to convenience and safety considerations. The maximum invasion efficiency of *E. coli* HB101 [16] was about 0.02%, however, the maximum invasion efficiency of *E. coli* #375 (inv^+) was 0.25%, an increase of approximately 12.5-fold over noninvasive *E. coli* HB101. In the study, the difference of the invasion efficiency between *E. coli* #375 (inv^+) and negative control *E. coli* HS was significant ($P < 0.01$). Therefore, *E. coli* #375 (inv^+) is likely to represent the invasion efficiency of *Yersinia*. The invasion efficiency of *E. coli* #375 (inv^+) was constant up to an MOI of 40, and then decreased sharply, likely reflecting saturation of host integrin receptors at higher MOIs.

Figure 1D shows that *E. coli* O157:H7 C8632 possessed an invasion efficiency of 2.8% at an MOI of 0.04, then linearly decreased invasion efficiency with further increasing MOIs. The invasion efficiency after an MOI of 4 was very low. This pattern is similar to the lower infectious dose (10 bacteria) of *E. coli* O157:H7 that has been estimated to cause diseases in clinical practice. In contrast, *C. freundii* 7004 and the noninvasive

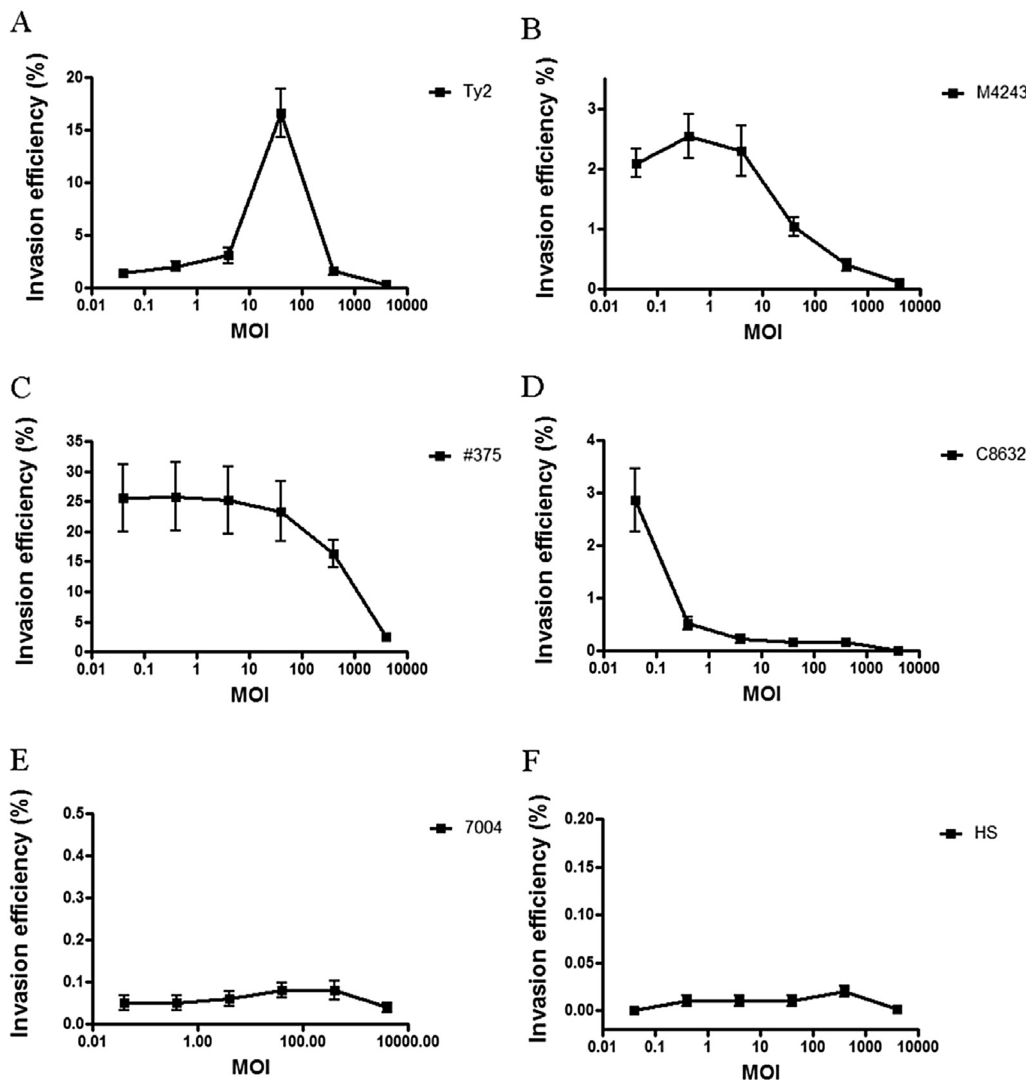

Figure 1. Efficiency of bacterial invasion into human INT407 cells at various starting MOIs.
(A) *S. typhi* Ty2; (B) *S. flexneri* 2a M4243; (C) *E. coli* #375 (inv^+); (D) *E. coli* O157:H7 C8632; (E) *C. freundii* 7004; (F) noninvasive control *E. coli* HS.

control *E. coli* HS exhibited very low invasion efficiency at all of the MOIs tested here (Figure 1E and F).

3.2. Patterns of internalized bacteria per well of cell culture plate in human INT407 cells

When the number of internalized bacteria resulting from varying the starting MOI is expressed as the number of internalized colony-forming unit averaged per cell culture plate well in a log-versus-log plot, an uphill curve was formed (Figure 2). *S. typhi* Ty2 at a starting MOI of 0.04 resulted in a weighted average of 66 internalized bacteria per well. This number increased to a peak at an average of about 400 000 bacteria per well (MOI of approximately 40) before invasion efficiencies decreased at higher MOIs (Figure 2A). This pattern was similar to that of a non-wild type strain *S. typhi* ty2w [16], but the invasion efficiency was much lower than that of *S. typhi* ty2w.

S. flexneri M4243, *E. coli* #375 (*inv*$^+$), or *C. freundii* 7004 at a starting MOI of 0.04 resulted in an average of 92, 102 or 3 internalized bacteria per well, respectively (Figure 2B–E). These numbers increased gradually with increasing MOIs. The higher the MOIs are, the more internalized bacteria per culture plate well increase.

Figure 2D and F show that the bacterial numbers of EHEC C8632 and *E. coli* HS per well appears to be increasing until an MOI of 400, and then decreasing at an MOI of 4 000. Therefore, the numbers of internalized bacteria did not continue to increase even though more bacteria were added.

3.3. Patterns of internalized bacteria per host cell in human INT407 cells

A curve or line resulted from monitoring the total number of bacteria per epithelial cell relative to various MOIs is shown in Figure 3. The number of internalized *S. typhi* Ty2 per host cell appeared to start increasing from an MOI of 4, and peaked at an MOI of 400, then decreased slowly (Figure 3A). The total numbers of colony-forming unit of internalized *S. typhi* remained relatively constant until to the MOI of 400, suggesting a stringent limitation on bacterial entry above MOIs of more than 400. Figure 3B shows

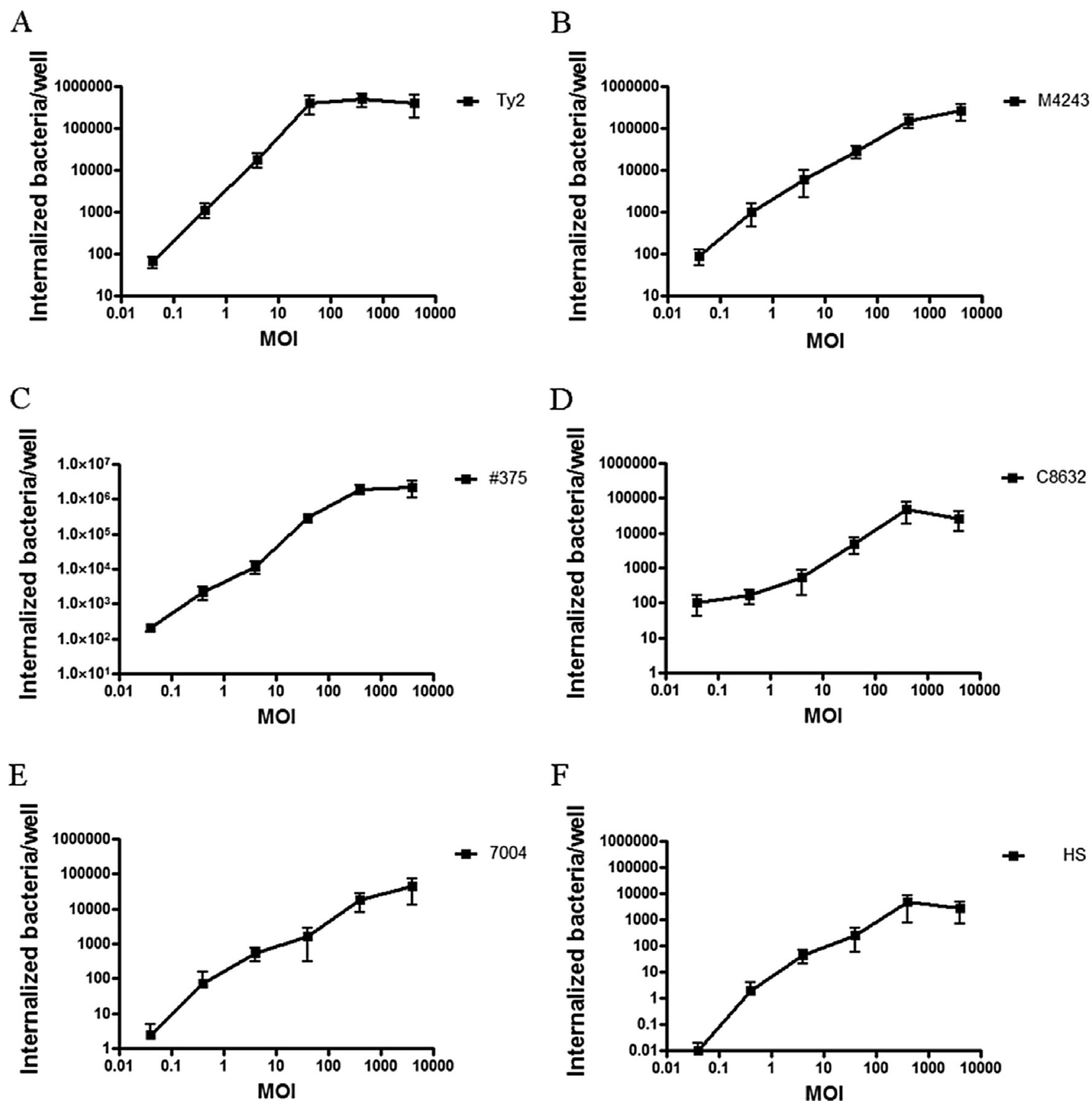

Figure 2. Number of bacteria internalized per well of cell culture plate at different starting MOIs.
(A) *S. typhi* Ty2; (B) *S. flexneri* 2a M4243; (C) *E. coli* #375 (*inv*$^+$); (D) *E. coli* O157:H7 C8632; (E) *C. freundii* 7004; (F) *E. coli* HS.

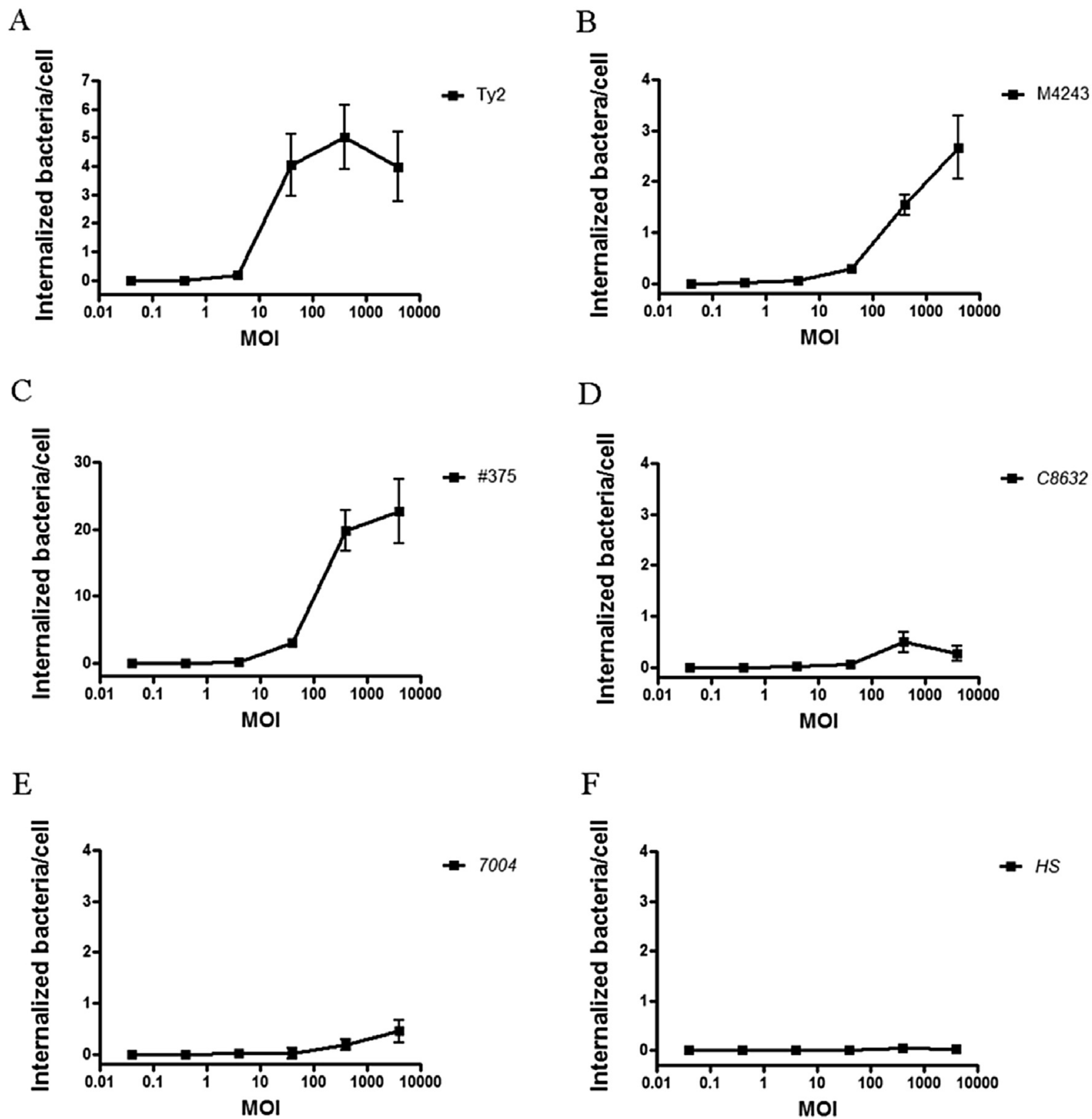

Figure 3. Number of bacteria internalized per host cell at different starting MOIs.
(A) *S. typhi* Ty2; (B) *S. flexneri* 2a M4243; (C) *E. coli* #375 (*inv*$^+$); (D) *E. coli* O157:H7 C8632; (E) *C. freundii* 7004; (F) *E. coli* HS.

that the number of the internalized *Shigella* per host cell started increasing from an MOI of 40, and then steadily kept the increase there after at all points measured. At an MOI of 4 000, there were about 3 bacterial cells/INT407 cell. Figure 3C shows that the number of *E. coli* 375 (*inv*$^+$) cells per host cell appeared to steadily increase after the MOI of 4.

In contrast, *E. coli* O157:H7C8632, *C. freundii* 7004, and *E. coli* HS internalized the INT407 cells at low and steady rates at most MOIs tested (Figure 3D–F).

3.4. Invasion effects of intestinal bacteria internalized into human HCT-8 cells

The HCT-8 cells were infected with the bacteria at various starting MOIs. Figure 4A shows that *E. coli* C8632 reached maximum invasion efficiency (approximately 2.6%) at an MOI of 0.04, and decreased gradually thereafter. The invasion efficiency of the organism arrived at the lowest at an MOI of 4 000. The higher the MOIs are, the lower the bacterial invasion efficiency is. The number of internalized EHEC cells per cell culture plate well increased gradually with increasing MOIs. With higher MOIs, more bacteria were internalized per host cell; at the MOI of 4 000, about 4.5 EHEC cells were internalized per host cell.

Similarly, *C. freundii* 7004 showed the highest invasion efficiency at an MOI of 0.04, but more bacteria were internalized at an MOI of 2 000. The noninvasive control *E. coli* HS showed a little higher invasion efficiency and internalized bacteria per host cell in the HCT-8 cells than those in INT407 cells.

3.5. Cytotoxicity assay

To investigate the detrimental effect of enteric bacteria on host cells, cytotoxicity assay was carried out according to the manufacturer's protocol. No significant differences were found between uninfected host cells and the infected host cells after the eukaryotic cells were incubated with/without the bacteria for 2 h (data not shown). The results suggested that little cytological and pathological damages resulted from the interaction of these pathogens with the host cells within the incubation period.

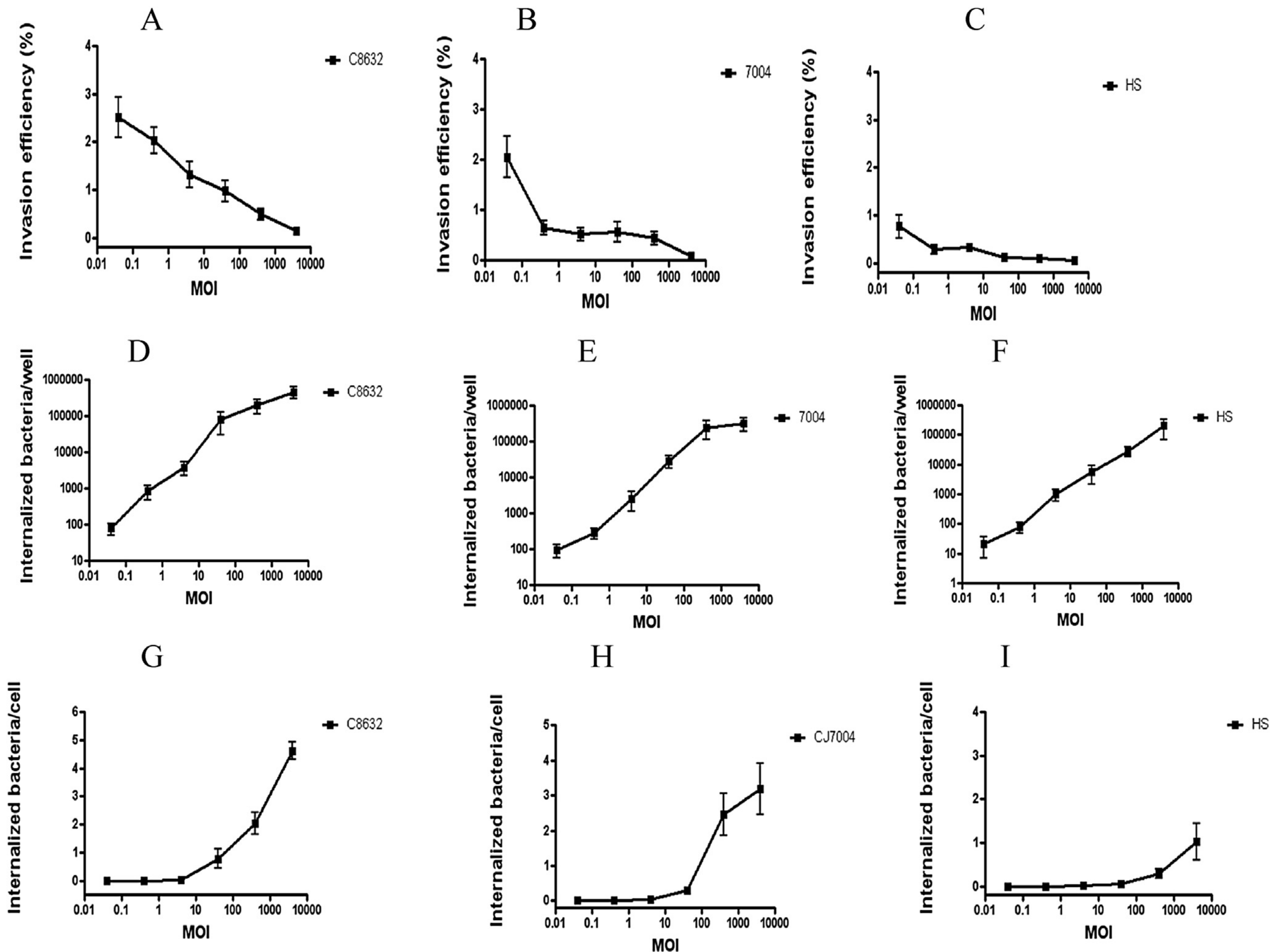

Figure 4. Comparative invasion kinetics for different MOIs with human HCT-8 cells.
(A), (B) and (C) show the bacterial invasion efficiency at different starting MOIs; (D), (E) and (F) present the total number of bacteria internalized in HCT-8 cells per well of cell culture plate at various starting MOIs. (G), (H) and (I) show the number of internalized bacteria averaged per HCT-8 cell at different starting MOIs. (A), (D) and (G): *E. coli* O157:H7 C8632; (B), (E) and (H): *C. freundii* 7004; (C), (F) and (I): *E. coli* HS.

4. Discussion

To be successful in causing disease, invasive bacterial pathogens employ different invasion mechanisms to internalize into eukaryotic intestinal epithelial cells. These bacterial pathogens detected here displayed markedly different invasion kinetics over the MOI ranges tested. The different invasion effects of these enteric bacteria may reflect their specific molecular invasion mechanisms and sensitivity to the hosts. Some bacterial pathogens such as *Salmonella* and *Shigella* invade epithelial cells through a Type III secretion system (T3SS), which is a molecular machine used to inject effector proteins into eukaryotic host cells. *Salmonella* pathogenicity island 1 contains approximate 30 genes involved in the formation of a T3SS and encodes multiple effector proteins. The effector proteins of *Salmonella typhimurium* T3SS-1 initiate the invasion of epithelial cells by mediating actin cytoskeleton rearrangements and membrane ruffling to trigger bacteria uptake [17–19]. The virulence of the human-specific pathogen *S. typhi* is not well understood because partially no suitable animal model is available. However, *S. typhi* shares a homolog T3SS-1 with *S. typhimurium* [20]. These two *Salmonella* serovars might share a similar invasion mechanism at a certain level. *Shigella* T3SS displays over 20 known effector proteins. The ensuing invasion process relies on the expression of the effector proteins which promote *Shigella* internalized into human epithelial cells [21,22].

The locus of enterocyte effacement pathogenicity island in *E. coli* O157:H7 encodes a T3SS, an adhesin (the intimin Eae), and its receptor (Tir) that are required for intimate adherence to intestinal epithelial cells [23]. These effector proteins are injected by the T3SS and trigger actin polymerization and microvilli effacement of the host cells [23–25]. However, some locus of enterocyte effacement-negative EHEC strains are also responsible for causing diseases [26]. *E. coli* O157:H7 typically produce Shiga-like toxins [27,28], and have a 60-MDa plasmid which encodes fimbriae that mediate bacterial attachment to cultured INT407 cells [29]. Although most EHEC were thought to be noninvasive, Oelschlaeger *et al.* [30] reported several *E. coli* O157:H7 isolates were able to invade human T24 bladder cells and HCT-8 epithelial cells at a substantial level. The invasive ability of ETEC cells varies among different serotypes [31]. Our study confirmed that *E. coli* O157 C8632 did invade HCT-8 cells.

Some invasive bacteria penetrate the host cell with the help of specific receptors. For example, the invasion of *Yersinia* in human epithelial cells is dependent on a protein called invasin encoded by a chromosomal *inv* gene. Invasin, the primary

invasion factor, binds with high affinity to β1 chain integrin receptors and mediates efficient and rapid internalization into host cells. It is reported that an *inv* mutant was unable to invade cultured epithelial cells as efficiently as wild type [32,33]. *Yersinia* Yop proteins encoded by a T3SS and the attachment invasion locus *ail* are also involved in the bacteria invasion and survival, as well as in inflammatory responses [34]. Type 1 pili and *de novo* protein of *C. freundii* mediate adherence and invasion into host cells [35,36]. The different invasion mechanisms may control the invasion effects of these bacteria.

Shigella species are highly infectious, since as few as 10 microorganisms are sufficient to cause diseases [11]. The low infectious dose can at least partially be attributed to the presence of an effective acid resistance system, which enables *S. flexneri* to survive the acidic environment of the stomach through an up-regulation of acid resistance genes [37]. Furthermore, it was shown that *Shigella* are able to down-regulate the expression of host antimicrobial peptides [38]. Although the invasion efficiency of *Yersinia* and *S. typhi* was higher than that of *Shigella*, their infectious doses were higher than those of *Shigella* too. It might result from lower resistance to gastric acid and bactericidal factors associated with the human defensive systems.

The bacterial sensitivity to host cells was also important and may involve in the effects of bacterial invasion. Some bacteria only invade specific eukaryotic cells while others invade a wide-range of host cells. EHEC 8632 and *C. freundii* 7004 have low invasion efficiency in INT407 cells; however, they have been reported to have higher invasion efficiency in human ileocecum HCT-8 cells [30]. So we used the HCT-8 cells to test invasion efficiency of *E. coli* O157:H7 8632 and *C. freundii* 7004. Our results demonstrated these two bacteria had higher invasion effects in the human HCT-8 cells. Thus, selection of the appropriate cultured eukaryotic cell lines for testing the bacterial invasion efficiency is crucial for getting reliable results.

Taking together, this study shows that these tested foodborne bacterial pathogens possess different invasive patterns at various starting MOIs. Higher starting MOIs did not always lead to more bacteria being internalized. These bacterial invasive effects also varied in different cell lines. In addition, our data confirmed that *E. coli* O157:H7 C8632 did invade human intestinal cells. These results can help to figure out the appropriate infectious doses of the bacterial pathogens in *in vitro* invasion assays, animal assays and vaccine clinical trials. Finally, the invasion kinetics of these pathogens is useful in designing and evaluating the safety and efficacy of live attenuated bacterial vaccines.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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