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NaCl Effect on Invasive *Staphylococcus aureus* Invasion and Immune Responses in the Intestinal Epithelial Cell

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Abstract

This study investigated the immune responses, which may cause chronic problems through intestinal invasion by *Staphylococcus aureus*, which is well-known to cause intoxication rather than invasion, and NaCl effect on the invasion. Five *S. aureus* strains were cultured and exposed to NaCl concentrations of 0%, 2%, 4%, or 6% supplemented in tryptic soy broth. The surviving cells were isolated by subsequent exposure of the culture on tryptic soy agar containing 0%, 2%, 4%, or 6% NaCl. The resulting cells were subjected to assays evaluating the invasion efficiency into Caco-2 cells, the level of immune responses generated, and the resultant cell viability. For *S. aureus* strains that invaded Caco-2 cells, the transcriptional analysis for adhesion related genes was conducted by quantitative real-time PCR. Only *S. aureus* ATCC14458 demonstrated obvious invasion into Caco-2 cells. Invasion efficiency of the strain was influenced by NaCl levels, and expression levels of the cell adhesion related genes were higher at 2%, 4%, and 6% NaCl. In addition, inflammation-related factors were increased. These results indicate that *S. aureus* ATCC14458 can invade Caco-2 cells, which may cause infectious illness and other chronic symptoms, and that NaCl increases the extent of Caco-2 cell invasion by expressing specific genes.

Keywords: Inflammation; Invasion; NaCl; *Staphylococcus aureus*

Introduction

Staphylococcus aureus is usually found in the human nose and skin [1], and thus can be cross-contaminated from humans to foods. *S. aureus* is weakly competitive in microflora, but it can proliferate in the range of pH 4.0-10.0, Aw >0.86 and even in high salt concentrations up to 10-15% [2]. Thus, *S. aureus* foodborne illnesses are usually related to salted foods as a result of enterotoxin generation and not by infection [3,4]. NaCl in foods increases the osmotic pressure, which may foodborne pathogens [5]. Biofilm production of *S. aureus* was increased by exposure to 3% NaCl [6]. In addition, some strains of *Listeria monocytogenes* exposed to between 2% and 4% NaCl generated slightly thicker biofilms of adherent bacteria [7].

Host cell invasion of bacteria occurs via several mechanisms. *Salmonella* spp. and *Shigella* spp. directly manipulate the host cytoskeleton by injecting active proteins intracellularly [8-10]. Yersinia uses a surface protein to bind integrin β1, which is a receptor expressed in the plasma membranes of host cells [11]. *L. monocytogenes* functions using a similar mechanism; specifically, the internalin A of the bacterium attaches to E-cadherin on the cell surface [10], and the internalin B binds the extracellular domain of c-Met [12]. Unlike these foodborne pathogens, *S. aureus* produces enterotoxins in foods at 10^5-10^6 CFU/g, resulting in intoxication foodborne illnesses [3,13]. If *S. aureus* is ingested with contaminated food at levels below 10^5CFU/g, there is no resultant symptom. However, the responses of *S. aureus* in the intestine have not been elucidated so far.

Therefore, the objective of this study was to identify if *S. aureus* can invade human intestinal epithelial cells (Caco-2 cell) and stimulate immune responses, and to characterize the effect of NaCl on the invasion process.

Materials and Methods

Cell culture

Caco-2 cells (KCLB 30037.1) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in Eagle's Minimum Essential Medium (MEM; Gibco, Penrose, Auckland, New Zealand) supplemented with 20% Fetal Bovine Serum (FBS; Gibco) and 1% Penicillin-Streptomycin (PS; Gibco). Media replacement was conducted every 2 to 3 days.

Preparation of inocula

Five *S. aureus* strains [NCCP10826 (SEC; staphylococcal enterotoxin C), ATCC13565 (SEA), ATCC14458 (SEB), ATCC23235 (SED), and ATCC27664 (SEE)] were cultured in tryptic soy broth (TSB; Becton Dickinson and Company, Franklin Lakes, NJ, USA) at 37°C for 24h, and 0.1mL of the culture was transferred into 10mL TSB. After incubation at 37°C for 24h, the cells were harvested by centrifugation (1,912×g, 4°C, and 15min), washed twice with phosphate buffered saline (PBS, pH 7.4; 0.2g of KH_2PO_4, 1.5g of $Na_2HPO_4 \cdot 7H_2O$, 8.0g of NaCl, and 0.2g of KCl in 1L of distilled water), and resuspended in PBS. The suspension was diluted in PBS to obtain 4log CFU/mL. The diluent (0.1mL) was then inoculated into 10mL TSB supplemented with 0%, 2%, 4%, or 6% NaCl and incubated overnight at 37°C. The 0.1mL volumes of each culture were plated on tryptic soy agar (TSA; Becton Dickinson and Company) containing 0%, 2%, 4%, or 6% NaCl, to obtain either non-NaCl stress adapted cells or NaCl stress adapted cells. After incubation at 37°C for 24h,

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Table 1: Oligonucleotide primers used in the quantitative real-time PCR analysis.

Gene	Primer	Sequence (5′→3′)
16s RNA	16s RNA-F	GAG GGT CAT TGG AAA CTG GA
	16s RNA-R	CAT TTC ACC GCT ACA CAT GG
icaA	icaA-F	CGC AGC AGT AGT TCT TGT CG
	icaA-R	TGA CCA TGT TGC GTA ACC AC
icaB	icaB-F	AAG CAG TCA CTC CGA ACT CC
	icaB-R	ATG GAA TCC GTC CCA TCT CT
icaC	icaC-F	TGG AAC GTT ACC AGC TTT TCA
	icaC-R	AAT GCG TGC AAA TAC CCA AG
icaD	icaD-F	TTG AAA CTA TGG GCA TTT TCG
	icaD-R	CAC GAT TCT CTT CCT TTC TGC
fnbA	fnbA-F	CTT TGG CAG GTG GTA CTG GT
	fnbA-R	GAG CCA GAA ACT CCA ACA CC

3mL PBS was added over the colonies, and the colonies were scraped with a glass rod. The collected *S. aureus* cells were then centrifuged (1,912×g, 4°C, 15min), and the pellets were then washed twice with PBS. These bacterial cell suspensions were then adjusted to OD_{600} = 0.02 for Caco-2 cell invasion assay or adjusted to OD_{600} = 0.1 for measuring immune responses and cell viability.

Caco-2 cell invasion assay

The 0.5mL volumes of the diluents for five *S. aureus* strains were inoculated into 4.5mL Eagle's minimum essential medium (MEM; Gibco, Penrose, Auckland, New Zealand) supplemented with 20% fetal bovine serum (FBS; Gibco), followed by gentle inversions. The 0.1mL volumes of these mixtures were plated on TSA to determine the initial populations of *S. aureus*. One-milliliter volumes of the mixtures were then inoculated into a monolayer of Caco-2 cells (5×10^4 cells/mL) in MEM + 20% FBS and incubated in 5% CO_2 at 37°C for 2h. The upper layer of MEM + 20% FBS was discarded, and the Caco-2 cells were further incubated in fresh MEM + 20% FBS or fresh MEM + 20% FBS along with 50μg/mL gentamicin to remove the *S. aureus* cells that were attached on Caco-2 cells in CO_2 at 37°C for 2h. After the incubation, the upper layer of the media was discarded, and the Caco-2 cells were washed with Dulbecco's phosphate buffered saline (DPBS; Welgene, Daegu, Korea) twice. A solution (1mL) of 0.5% Triton X-100 (Sigma-Aldrich Co., St. Louis, State of Missouri, USA) was then added into each well, and the microtiter plate was left on ice for 20min. The resulting suspension (0.1mL) was plated on TSA to enumerate invaded (gentamicin treated group) and attached *S. aureus* populations (non-gentamicin treated group-gentamicin treated group). The efficiency of *S. aureus* invasion of Caco-2 cells was calculated by the equation [1] as follows:

Invasion efficiency (%) = [number of *S. aureus* cells invading Caco-2 cells (CFU/mL)]

× [(initial cell counts of *S. aureus* {CFU/mL})$^{-1}$]×100 [1]

Immune response analysis

After evaluating the extent of Caco-2 cell invasion, the invasive strain (*S. aureus* ATCC14458) was selected. Invasive *S. aureus* strains and non-invasive *S. aureus* strains (NCCP10826 and ATCC13565) were subjected to immune response analysis. The *S. aureus* strains were diluted with MEM to a concentration of 5×10^6 CFU/mL, and 1mL volumes of diluents were inoculated into 5×10^4 cells/mL of Caco-2 cells. They were then incubated in 5% CO_2 at 37°C for 24h, followed by centrifugation. After centrifugation, the supernatant was used for immune response analysis with Luminex® extracellular assay (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's protocols.

Table 2: Adhesion efficiency (mean ± standard deviation) of *Staphylococcus aureus* strains NCCP10826, ATCC13565, ATCC14458, ATCC23235, and ATCC27664, which were exposed to tryptic soy agar plus 0, 2, 4, and 6% NaCl.

Strain	NaCl concentration (%)			
	0	2	4	6
10826	0.004±0.000 ^{Ab}	0.002±0.000 ^{Ab}	0.001±0.000 ^{Ab}	0.001±0.000 ^{Ab}
13565	0.011±0.012 ^{Ab}	0.015±0.011 ^{Ab}	0.029±0.040 ^{Ab}	0.020±0.023 ^{Ab}
14458	1.887±1.124 ^{Ca}	6.443±3.153 ^{Aa}	4.010±1.908 ^{Ba}	3.269±0.874 ^{BCa}
23235	0.005±0.001 ^{Ab}	0.012±0.002 ^{Ab}	0.001±0.001 ^{Ab}	0.001±0.000 ^{Ab}
27664	0.015±0.020 ^{Ab}	0.015±0.006 ^{Ab}	0.000±0.000 ^{Ab}	0.005±0.007 ^{Ab}

$^{A-C}$Means within the same row with different superscript letters are significantly different ($p<0.05$).
$^{a-b}$Means within the same column with different superscript letters are significantly different ($p<0.05$).

Table 3: Invasion efficiency (mean ± standard deviation) of *Staphylococcus aureus* strains NCCP10826, ATCC13565, ATCC14458, ATCC23235, and ATCC27664, which were exposed to tryptic soy agar plus 0, 2, 4, and 6% NaCl.

Strain	NaCl concentration (%)			
	0	2	4	6
10826	0.005±0.000 ^{Ab}	0.017±0.004 ^{Ab}	0.005±0.000 ^{Ab}	0.037±0.010 ^{Ab}
13565	0.025±0.015 ^{Ab}	0.035±0.001 ^{Ab}	0.010±0.008 ^{Ab}	0.097±0.074 ^{Ab}
14458	3.968±0.834 ^{Ca}	5.480±3.281 ^{BCa}	5.847±1.590 ^{Ba}	9.097±1.241 ^{Aa}
23235	0.174±0.052 ^{Ab}	0.216±0.046 ^{Ab}	0.099±0.061 ^{Ab}	0.191±0.132 ^{Ab}
27664	0.237±0.271 ^{Ab}	0.263±0.079 ^{Ab}	0.089±0.061 ^{Ab}	0.157±0.022 ^{Ab}

$^{A-C}$Means within the same row with different superscript letters are significantly different ($p<0.05$).
$^{a-b}$Means within the same column with different superscript letters are significantly different ($p<0.05$).

Cell viability assay

A total of 20μl of the diluents of either invasive *S. aureus* strain ATCC14458 or the non-invasive *S. aureus* strains were inoculated into 180μl MEM supplemented with 20% FBS in 5% CO_2 at 37°C for 24h. The upper layer of MEM + 20% FBS was discarded, and the Caco-2 cells were further incubated in fresh MEM + 20% FBS along with 100μg/mL gentamicin to extricate the previously attached *S. aureus* cells from Caco-2 cells at 37°C for 2h. After the incubation, the upper layer of the media was discarded, and the 200μl cell media with 20μl of thiazoly blue tetrazolium bromide (Sigma-Aldrich Co., St. Louis, State of Missouri, USA) solution (5mg/ml in dH_2O) was added to each well for 1h. After the incubation, the upper layer of the media was discarded, and 200μl of dimethyl sulfoxide (DMSO; Samchun pure chemical Co., LTD., Pyeongtaek, Korea) was added into each well. Optical density was then measured at 540nm.

Transcriptome analysis

The OD_{600} value of NaCl stress adapted cells (*S. aureus* strains ATCC14458, NCCP10826, and ATCC13565) was calibrated to 0.5 with PBS. After centrifuging 1.5mL of the cultures (5,000×g, room

Table 4: Cytokines, chemokines, and growth factors stimulated by invasive *Staphylococcus aureus* strain (ATCC14458), and non-invasive *S. aureus* strains (NCCP10826 and ATCC13565).

Group	Analyte name	MEM blank (pg/ml)	MEM +cell blank (pg/ml)	ATCC14458	NCCP10826	ATCC13565
Cytokines	Flt-3 Ligand	2	7	64±10 A	10±10 B	6±8 B
	G-CSF	4	6	57±0 A	13±1 C	25±3 B
	GM-CSF	0	0	38±9 A	1±1 B	1±1 B
	IFNα2	0	0	60±2 A	5±6 B	2±1 B
	IFNγ	0	0	107±10 A	6±5 B	1±2 B
	IL-1α	0	0	1±0 A	0±0 B	0±0 B
	IL-1β	0	1	6±0 A	0±0 B	0±1 B
	IL-1γα	1	1	17±3 A	3±1 B	1±1 B
	IL-2	0	0	6±1 A	0±0 B	0±0 B
	IL-3	0	0	43±4 A	1±1 B	1±1 B
	IL-4	2	5	16±2 A	4±2 B	8±2 B
	IL-5	0	1	2±0 A	1±0 A	1±1 A
	IL-6	0	0	32±1 A	3±1 B	2±1 B
	IL-7	0	0	9±0 A	2±3 A	3±4 A
	IL-8	1	18	23±1 C	64±0 A	49±2 B
	IL-9	0	0	24±3 A	2±0 B	2±0 B
	IL-10	0	0	6±3 B	0±0 A	1±0 A
	IL-12(p40)	0	0	56±3 A	1±1 B	0±0 B
	IL-12(p70)	2	3	256±6 A	12±3 B	14±4 B
	IL-13	0	0	6±1 A	0±0 B	0±0 B
	IL-15	1	1	10±1 A	2±0 B	1±0 B
	IL-17	0	0	25±0 A	1±0 B	2±1 B
	TNFα	0	1	6±0 A	1±0 B	0±0 B
	TNFβ	0	0	2±3 A	0±0 A	1±1 A
Chemokines	Eotaxin	9	5	55± 1 A	17±4 B	20±4 B
	Fractalkine (FKN)	5	0	135±11 A	25±21 B	61±3 B
	IP-10	0	84	107±4 B	231±10 A	222±41 A
	MCP-1	1	6	10±2 A	12±0 A	10±2 A
	MCP-3	8	16	145±11 A	24±4 B	30±11 B
	MDC	0	3	14±2 A	6±0 B	0±0 C
	MIP-1α	6	4	28±2 A	5±7 B	0±0 B
	MIP-1β	0	0	23±1 A	0±0 B	5±3 B
	RANTES	3	4	8±0 A	6±1 A	3±4 A
Growth factors	EGF	1	1	27±2 A	6±4 B	5±3 B
	FGF-2	9	7	382±22 A	43±11 B	32±6 B
	GRO	0	269	591±54 B	962±17 A	854±94 A
	PDGF-AA	0	235	112±12 B	161±1 AB	169±28 A
	PDGF-AB/BB	13	8	236±37 A	20±4 B	17±3 B
	TGFα	0	0	2±0 A	0±0 B	0±0 B
	VEGF	0	64	334±32 A	82±44 B	0±0 B

A-C Means with the same row with different superscript letters are significantly different ($p<0.05$).

temperature, and 5min), the supernatants were discarded, and 200μL of lysostaphin (200μg/mL) (Sigma-Aldrich Co.) was added to the pellets and incubated at 37°C for 20min. By following the manufacturer's instructions, RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration of total RNA was measured using the Epoch Micro-Volume Spectrophotometer System (Bio Tek Instruments, Winooski, VT, USA). Complementary DNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocols. The PCR reaction mixture (25μL) was prepared with the Rotor-Gene SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. The relative expression levels of the genes were analyzed with Rotor-Gene Q software (Qiagen) to compare the expression levels of the genes related to cell adhesion in *S. aureus* grown in the absence or presence of NaCl. Analysis of the gene expression levels was performed in duplicate per replication, and more than two-fold increases were considered significant [14]. To evaluate the relative expression levels, the primers of the genes are listed in Table 1.

Statistical analysis

The experiment was repeated twice with four samples per repeat (n=8). The experimental data for the quantitative analysis was analyzed with the general linear procedure of SAS® version 9.2 (SAS Institute Inc., Cary, NC, USA). The mean comparisons were performed by a pairwise t-test at $\alpha = 0.05$.

Results

Caco-2 cell invasion and immune response

Of the five *S. aureus* strains examined, *S. aureus* ATCC14458 had higher ($p<0.05$) adhesion efficiency (1.887-6.443 %) on Caco-2 cells than the other strains (0.000-0.029 %) (Table 2). In addition, only *S. aureus* ATCC14458 (3.968-9.097 %) showed obvious Caco-2 cell invasion compared to the other strains (0.005-0.263 %), and the Caco-2 cell invasion efficiency of the strain was increased ($p<0.05$) as the NaCl concentration that *S. aureus* ATCC14458 was exposed to increased (Table 3). To confirm the inflammation, *S. aureus* ATCC14458 was subjected to immune response analysis and the levels of 40 cytokines, chemokines, and growth factors were measured. The cytokines, chemokines, and growth factors generated by *S. aureus* ATCC14458 or non-invasive strains (NCCP10826 and ATCC13565) were measured. Especially, IFNγ was increased ($p<0.05$) in invasive *S. aureus* strain by protective inflammatory reaction in Caco-2 cell. Interestingly, the Caco-2 cells inoculated with invasive *S. aureus* strain exhibited higher ($p<0.05$) production of Fibroblast Growth Factor-2 (FGF-2), Fractalkine (FKN), IL-12 (p70), monocyte chemoattractant protein-3 (MCP-3), platelet derived growth factor-AB/BB (PDGF-AB/BB), and Vascular Endothelial Growth Factor (VEGF) than non-invasive strains (Table 4). Although it was observed that *S. aureus* ATCC14458 can elicit the production of inflammatory cytokines, chemokines, and growth factors the concentration of NaCl employed did not affect the levels of cytokines secreted (Table 5).

Caco-2 cell viability analysis

To evaluate if invasive *S. aureus* can cause cytotoxicity in Caco-2 cells, an MTT assay was conducted [15]. The MTT assay demonstrated that there is no difference in cytotoxicity among the strains ($p>0.05$),

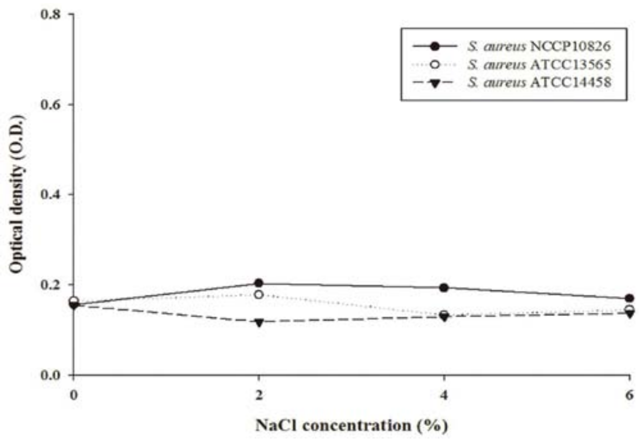

Figure 1: Cytotoxicity of NaCl (0%, 2%, 4%, and 6%)-adapted invasive *Staphylococcus aureus* strain (ATCC14458), and non-invasive *S. aureus* strains (NCCP10826 and ATCC13565) on Caco-2 cells.

regardless of NaCl concentrations (Figure 1). This result indicates that even though invasive *S. aureus* strains invade intestinal Caco-2 cell, the actual process of invasion itself does not cause cytotoxicity. Accordingly, we hypothesize that while the inflammation and other clinical symptoms immediately ensuing occur without resulting in intestinal cell damage, the symptoms may gradually develop to trigger disease in the long term.

Transcriptome analysis

To evaluate the correlation between the genes influenced by NaCl concentrations and *S. aureus* Caco-2 cell invasion, the expression levels of icaA, icaB, icaC, icaD, and fnbA were analyzed using qRT-PCR after *S. aureus* was exposed to 0%, 2%, 4%, or 6% NaCl. icaA, icaB, icaC, and icaD are the genes included in ica operon, which plays a role in polysaccharide intercellular adhesion [16]. fnbA is the gene encoding fibronectin-binding protein, which mediates bacterial adherence and coagulase [17]. Notably, the expression levels of all genes tested were higher in the invasive *S. aureus* strain, as compared to those in the non-invasive strains. The expression levels were also increased at higher NaCl concentration in *S. aureus* ATCC14458. Especially, the expression level of fnbA was increased by more than two-fold at 2%, 4%, and 6% NaCl, compared to that of 0% NaCl ($p<0.05$) (Figure 2A). This result suggests that the NaCl in food upregulates the ica operon and fnbA of invasive *S. aureus* strains, and that its attachment is regulated by polysaccharide intercellular adhesion, which may increase intercellular invasion. However, the expression levels of these genes were not increased in non-invasive *S. aureus* strains (Figure 2B and 2C).

Discussion

Caco-2 cell invasion and immune response data indicates that even though *S. aureus* is known to cause foodborne illnesses due to the consumption of enterotoxins produced by the pathogens in food [3], *S. aureus* ATCC14458 strain may cause infection in intestines, resulting in inflammation. NaCl in food may increase its infection because the genes are sensitively upregulated in *S. aureus* ATCC14458, which could be a major cause making the strain invasive. The Caco-2 cells inoculated with invasive *S. aureus* strains exhibited higher

Table 5: Cytokines, chemokines, and growth factors stimulated by invasive *Staphylococcus aureus* strain (ATCC14458) experienced 0, 2, 4, and 6% NaCl.

Group	Analyte name	MEM blank (pg/ml)	MEM +cell blank (pg/ml)	0%	2%	4%	6%
Cytokines	Flt-3 Ligand	0	0	38±13 ^{A}	23±3 ^{A}	29±0 ^{A}	31±1 ^{A}
	G-CSF	0	0	5±7 ^{A}	0±0 ^{A}	0±0 ^{A}	2±3 ^{A}
	GM-CSF	3	3	20±8 ^{A}	11±2 ^{A}	12±0 ^{A}	13±0 ^{A}
	IFNα2	0	0	3±3 ^{A}	0±0 ^{A}	2±1 ^{A}	1±0 ^{A}
	IFNγ	0	0	13±5 ^{AB}	8±1 ^{B}	17±2 ^{A}	18±1 ^{A}
	IL-1α	0	0	2±1 ^{A}	0±0 ^{A}	2±1 ^{A}	1±1 ^{A}
	IL-1β	2	2	13±5 ^{A}	8±0 ^{A}	9±0 ^{A}	8±0 ^{A}
	IL-1γα	0	0	32±16 ^{A}	20±0 ^{A}	16±6 ^{A}	19±5 ^{A}
	IL-2	0	0	22±9 ^{A}	10±1 ^{A}	13±1 ^{A}	12±1 ^{A}
	IL-3	1	1	14±0 ^{A}	5±0 ^{B}	5±1 ^{B}	5±1 ^{B}
	IL-4	0	0	3±4 ^{A}	1±0 ^{A}	1±1 ^{A}	1±1 ^{A}
	IL-5	1	1	1±0 ^{A}	1±0 ^{A}	1±0 ^{A}	1±0 ^{A}
	IL-6	0	0	11±4 ^{A}	5±0 ^{B}	6±0 ^{AB}	6±0 ^{AB}
	IL-7	1	1	2±0 ^{A}	1±1 ^{A}	2±0 ^{A}	2±0 ^{A}
	IL-8	3	6	11±1 ^{A}	9±2 ^{A}	13±0 ^{A}	15±0 ^{A}
	IL-9	2	2	20±10 ^{A}	11±1 ^{A}	13±0 ^{A}	12±0 ^{A}
	IL-10	0	0	0±0 ^{A}	0±0 ^{A}	0±0 ^{A}	0±0 ^{A}
	IL-12(p40)	0	0	108±44 ^{A}	58±10 ^{A}	70±8 ^{A}	72±1 ^{A}
	IL-12(p70)	2	2	82±25 ^{A}	34±3 ^{B}	45±4 ^{B}	51±0 ^{AB}
	IL-13	2	2	3±0 ^{A}	2±0 ^{A}	3±0 ^{A}	3±0 ^{A}
	IL-15	4	4	12±2 ^{A}	9±1 ^{A}	9±1 ^{A}	9±0 ^{A}
	IL-17	1	0	8±1 ^{A}	5±2 ^{A}	10±1 ^{A}	11±1 ^{A}
	TNFα	0	0	1±1 ^{A}	0±0 ^{A}	0±0 ^{A}	0±0 ^{A}
	TNFβ	0	0	0±0 ^{A}	0±0 ^{A}	0±0 ^{A}	0±0 ^{A}
Chemokines	Eotaxin	9	7	49±10 ^{A}	34±1 ^{A}	41±3 ^{A}	42±0 ^{A}
	Fractalkine (FKN)	0	0	136±47 ^{A}	77±19 ^{A}	126±16 ^{A}	147±46 ^{A}
	IP-10	5	27	31±2 ^{A}	26±1 ^{A}	29±1 ^{A}	31±4 ^{A}
	MCP-1	0	0	9±1 ^{A}	6±0 ^{A}	8±1 ^{A}	7±0 ^{A}
	MCP-3	9	4	56±12 ^{A}	32±3 ^{B}	43±3 ^{AB}	47±2 ^{AB}
	MDC	7	5	22±5 ^{AB}	16±2 ^{B}	28±4 ^{A}	30±3 ^{A}
	MIP-1α	8	5	16±12 ^{A}	10±3 ^{A}	19±2 ^{A}	19±2 ^{A}
	MIP-1β	0	0	14±4 ^{A}	7±0 ^{B}	9±1 ^{AB}	8±1 ^{AB}
	RANTES	5	4	6±0 ^{A}	6±0 ^{A}	5±0 ^{A}	6±1 ^{A}
Growth factors	EGF	3	1	60±25 ^{A}	37±3 ^{A}	46±3 ^{A}	50±0 ^{A}
	FGF-2	0	0	777±215 ^{A}	457±47 ^{B}	529±24 ^{AB}	549±18 ^{AB}
	GRO	0	134	214±13 ^{A}	191±4 ^{A}	198±21 ^{A}	226±18 ^{A}
	PDGF-AA	0	68	37±0 ^{A}	32±2 ^{A}	32±1 ^{A}	34±2 ^{A}
	PDGF-AB/BB	0	18	156±34 ^{A}	99±4 ^{B}	155±9 ^{A}	185±14 ^{A}
	TGFα	1	1	2±1 ^{A}	1±0 ^{A}	2±0 ^{A}	2±0 ^{A}
	VEGF	0	38	103±24 ^{AB}	66±3 ^{B}	104±0 ^{A}	115±10 ^{A}

$^{A\text{-}B}$Means with the same row with different superscript letters are significantly different ($p<0.05$).

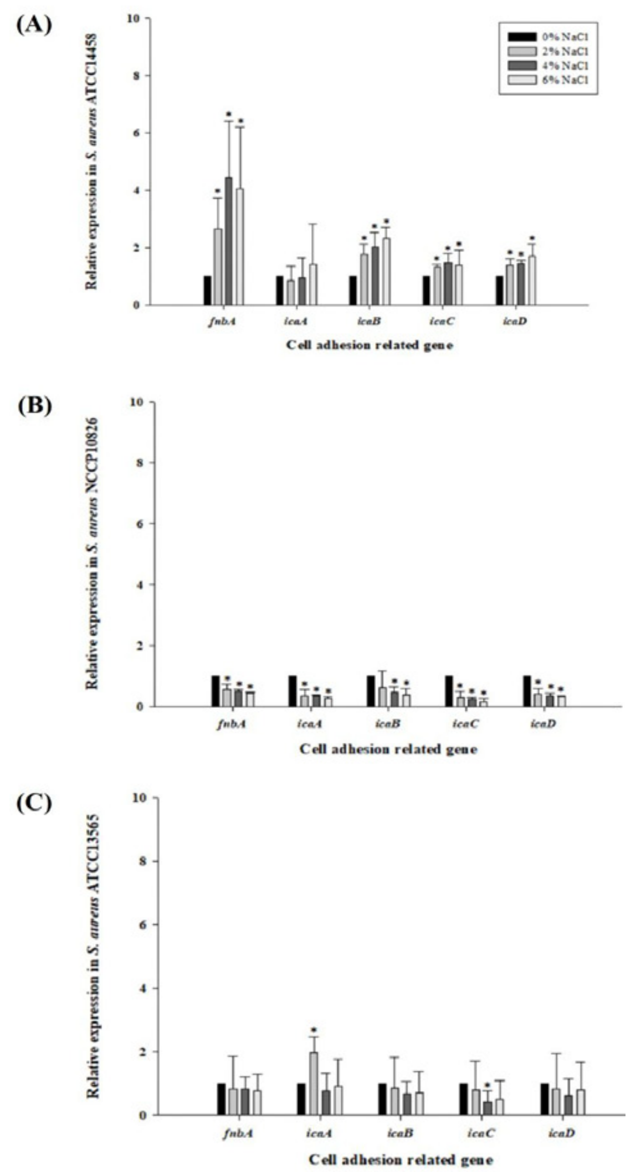

Figure 2: Relative expression of cell adhesion related genes after invasive *Staphylococcus aureus* strain (ATCC14458; A) and non-invasive *S. aureus* strains (NCCP10826; B and ATCC13565; C) were exposed to 0%, 2%, 4%, and 6% NaCl. *: Statistical significance composed to 0% NaCl at α=0.05.

production of some cytokines, chemokines, and growth factors as a part of protective mechanisms in the host cell [18,19]. FGF-2 plays a role in protecting cells from necrosis [20]. Further, FKN was secreted under inflammatory conditions in endothelial cells to coordinate leukocyte trafficking and thus, FKN expression is related to various inflammatory diseases [21,22]. In addition, it promotes the progression of cancer by supporting tumor angiogenesis [23]. The expression level of PDGF is low or undetectable in normal adult tissues, but its level is increased following tissue damage [24]. IL-12, produced by macrophages and B cells is also a powerful immunopotentiating agent. When IL-12 was administered to BALB/c mice infected with Mycobacterium tuberculosis, their survival time increased and there was a concomitant reduction in the number of bacteria present [25]. Moreover, IL-12 increased resistance to several intracellular pathogens in murine models. IL-12 has about seven times higher expression in breast tumor tissue as compared to normal tissue, and it plays an important role in the control of early stages of breast cancer [26] and inflammation response [27]. VEGF is known to play an important role as a stimulant in cancer angiogenesis [28]. Also, VEGF itself is not inflammatory, but it modulates the overall immune response associated with inflammation [29]. Taken together, *S. aureus* ATCC14458 may invade Caco-2 cells, resulting in the increased production of cytokines related to inflammation, chemokines, and growth factors. However, we found no differences in cell viabilities between invasive and non-invasive strains. Coussens and Werb [30] suggested that long-term inflammation in intestine may cause carcinogenesis. Thus, it can be suggested that although *S. aureus* invasion in intestine did not cause acute symptoms, but the inflammation caused by *S. aureus* invasion may cause chronic problems in long term. These results indicate that intake of *S. aureus* likely causes infection and potentially promotes microenvironmental conditions that are conducive to the development of various diseases. Therefore, further study is needed to elucidate the correlation between the *S. aureus* invasion and chronic disease development.

In conclusion, although *S. aureus* has been well established as a cause of intoxication foodborne illnesses, a *S. aureus* strain having high ica operon and fnbA gene expression can be ingested through food consumption and invade intestinal epithelial cells, and this may induce inflammation and chronic diseases in long term. In addition, the invasive efficiency is increased as the NaCl concentrations that *S. aureus* are exposed to in food increase.

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