Quantitative Microbial Risk Assessment for *Clostridium perfringens* in Natural and Processed Cheeses

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ABSTRACT: This study evaluated the risk of *Clostridium perfringens* (*C. perfringens*) foodborne illness from natural and processed cheeses. Microbial risk assessment in this study was conducted according to four steps: hazard identification, hazard characterization, exposure assessment, and risk characterization. The hazard identification of *C. perfringens* on cheese was identified through literature, and dose response models were utilized for hazard characterization of the pathogen. For exposure assessment, the prevalence of *C. perfringens*, storage temperatures, storage time, and annual amounts of cheese consumption were surveyed. Eventually, a simulation model was developed using the collected data and the simulation result was used to estimate the probability of *C. perfringens* foodborne illness by cheese consumption with @RISK. *C. perfringens* was determined to be low risk on cheese based on hazard identification, and the exponential model (r = 1.82×10^{-11}) was deemed appropriate for hazard characterization. Annual amounts of natural and processed cheese consumption were 12.40±19.43 g and 19.46±14.39 g, respectively. Since the contamination levels of *C. perfringens* on natural (0.30 Log CFU/g) and processed cheeses (0.45 Log CFU/g) were below the detection limit, the initial contamination levels of natural and processed cheeses were estimated by beta distribution ($\alpha_1 = 1$, $\alpha_2 = 91$; $\alpha_1 = 1$, $\alpha_2 = 309$)×uniform distribution (a = 0, b = 2; a = 0, b = 2.8) to be –2.35 and –2.73 Log CFU/g, respectively. Moreover, no growth of *C. perfringens* was observed for exposure assessment to simulated conditions of distribution and storage. These data were used for risk characterization by a simulation model, and the mean values of the probability of *C. perfringens* foodborne illness by cheese consumption per person per day for natural and processed cheeses were 9.57×10^{-14} and 3.58×10^{-14}, respectively. These results indicate that probability of *C. perfringens* foodborne illness by consumption cheese is low, and it can be used to establish microbial criteria for *C. perfringens* on natural and processed cheeses. (**Key Words:** Quantitative Microbial Risk Assessment, *Clostridium perfringens*, Cheese)

INTRODUCTION

Clostridium perfringens (*C. perfringens*) is a Gram-positive, non-motile, spore forming pathogen, and an anaerobic bacterium. The pathogen is usually isolated from soil, dust, water, food, and the gastrointestinal tract of humans and animals (Juneja et al., 2003; 2009). Spores of *C. perfringens* are widely distributed in nature, and its spores survive for several years and germinate at optimal growth temperature (Labbe and Juneja, 2006). The spores have a resistance to heat, and can resist up to 100°C for 1 h (Labbe, 2001; Byrne et al., 2006). This spore can even survive during cooking processes (Sarker et al., 2000). Hence, it is critical

to control *C. perfringens* in food processing, and to avoid inadequate cooling especially when cooking foods (Le Marc et al., 2008).

Various types of cheeses have been associated with foodborne illnesses related to *Listeria monocytogenes*, *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus* (Little et al., 2008; Kousta et al., 2010). However, foodborne illness by *C. perfringens* in cheeses have not been reported, even though one million people are affected by *C. perfringens* every year (Grass et al., 2013). The number of foodborne illness from *C. perfringens* might be underestimated because the symptoms of *C. perfringens* foodborne illness are mild and most of the symptoms last only 24 h (Schneider et al., 2014). However, Feligini et al. (2014) found that *C. perfringens* was recovered from 98.7% and 100% of milk and curds of Grana Padano cheese,

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respectively, indicating that the pathogen in cheese containing high moisture concentration may cause foodborne illness.

In Korea, cheese product falls into two categories; natural cheese and processed cheese. Natural cheese refers to as that manufacturing by removing the whey after coagulation of raw milk or milk product through addition of lactic acid bacteria, rennet and organic acid, and processed cheese is defined as containing more than 50% milk solid derived from natural cheese (MFDS, 2014a). Korea applies very strict microbial criteria (n = 5, c = 0, m = 0 for 25 g) for *C. perfringens*, but many countries do not have a microbial criteria for *C. perfringens* in cheese. Hence, scientific evidence is necessary to evaluate if the regulation in Korea is appropriate.

Quantitative microbial risk assessment (QMRA) has been used to estimate the risk of foodborne illness in many countries (Heidinger et al., 2009). Especially, ready-to-eat foods including cheese were assessed for microbial risk of *L. monocytogenes* (FDA-CFSAN and USDA-FSIS, 2003). In addition, Bemrah et al. (1998) and Farber et al. (1996) also conducted QMRA for human listeriosis in soft cheese. Recently, to establish the microbial criteria, microbial risk assessment has been necessary. Hence, the QMRA can be useful in establishing microbial criteria for *C. perfringens* in cheese. Therefore, the objective of this study was to develop a microbial risk assessment simulation model and to estimate the risk of *C. perfringens* in natural and processed cheese (Figure 1).

MATERIALS AND METHODS

Harzard identification

Hazard identification is defined by Codex (1999) as "the identification of biological, chemical, and physical agents capable of causing adverse health effects and which may be present in a particular food or group of foods". *C. perfringens* is a pathogenic bacterium for humans and animals, and it causes vomiting, abdominal cramps, and diarrhea within 24 h. The pathogen was classified into one of five types (A-E) according to the production of four major toxins (alpha, beta, epsilon, and iota) (Gao and McClane, 2012). Less than 5% of *C. perfringens* type A isolates produce another toxin named *C. perfringens* enterotoxin (CPE), which causes symptoms associated with several common gastrointestinal diseases (McClane, 2007; Gao and McClane, 2012). CPE can change membrane permeability which causes loss of H_2O, Na^+, and Cl^-, and these mechanisms produce the symptoms of foodborne illness (Brynestad and Granum, 2002). Although *C. perfringens* is an anaerobic bacterium, the pathogen can produce spores which can survive a variety of lethal factors such as heat, prolonged refrigeration and frozen temperatures, chemicals, and high hydrostatic pressure (Sarker et al., 2000; Li and McClane, 2006a; b; Paredes-Sabja et al., 2007). In addition, *C. perfringens* spores can survive thermal processing and sanitizing treatments
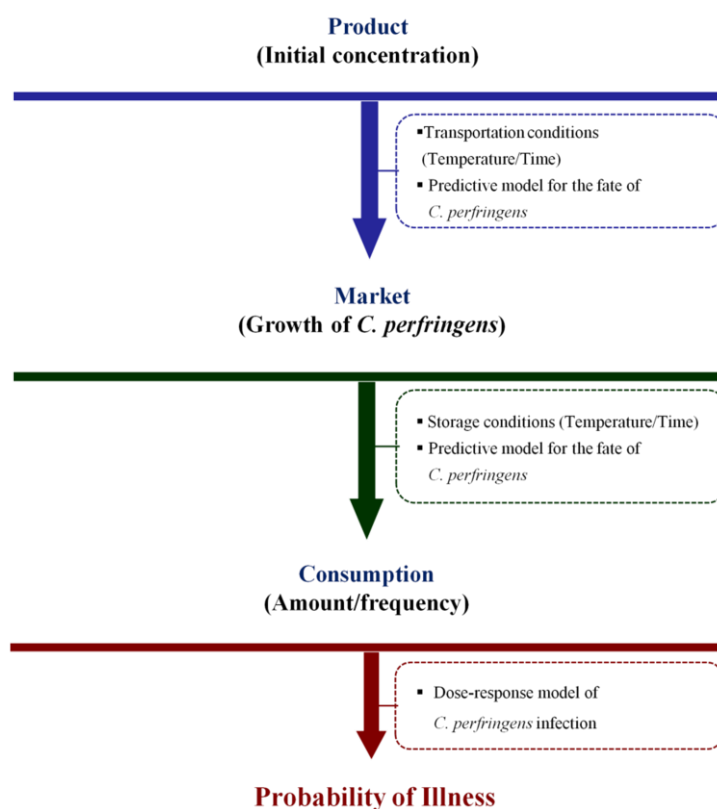

Figure 1. Flow chart of quantitative microbial risk assessment of *Clostridium perfringens* in natural and processed cheeses.

employed in the food industry (Udompijitkul et al., 2013). The spores can germinate under appropriate conditions such as 33°C to 49°C temperatures, and might cause foodborne illness.

In the U.S., *C. perfringens* is a common cause of foodborne illness with 1,461 cases of food illness, resulting in 1 hospitalization in 2014 (CDC, 2016). In Korea, *C. perfringens* caused 47 outbreaks of foodborne illness, and led to 2,686 hospitalizations in the last decade (MFDS, 2014b).

Although cheeses were considered one of the safe foods, *C. perfringens* might grow because of the physicochemical characteristics of cheese even in vacuum packaging condition. Thus, QMRA for *C. perfringens* in cheese is necessary.

Exposure assessment

Prevalence: *C. perfringens* prevalence and contamination levels were evaluated in two different cheese factories during the summer and winter. Natural cheese samples were collected from raw milk, pasteurized milk, cheese before ripening, cheese after packaging, and cheese before shipping. Since processed cheeses were manufactured from natural cheeses, samples were collected from the natural cheese used for the processed cheese, cheese after packaging and cheese before shipping. The collected samples were transported in an ice cooler to a laboratory, and milk (raw milk and pasteurized milk; 1 mL) samples were plated on tryptic soy agar (TSA; Difco, Sparks, MD, USA) and tryptose sulphite cycloserine agar (TSC; Oxoid Ltd, Basingstoke, Hampshire, England), followed by overlaying 10 mL egg yolk free TSC over the TSC to enumerate total bacterial and *C. perfringens* cell counts, respectively. The cheeses samples (natural cheeses used for processed cheese, before ripening, after packaging, and before shipping; 25 g or 1 slice) were aseptically transferred into a sample bag (3M St. Paul, MN, USA), and 50 mL of 0.1% buffered peptone water (BPW; Difco, USA) were added to the sample bag and pummeled for 120 s (BagMixer, Interscience, St. Nom, France). One milliliter of homogenate was then surface-plated on TSA and TSC, followed by overlaying 10 mL egg yolk free TSC over the TSC, respectively. The colonies were counted manually after incubation at 35°C for 24 h. Presumptive colonies of *C. perfringens* were further analyzed by 16s rDNA analysis to identify *C. perfringens*. To amplify 16s rDNA, primers of 27F (5′ – AGA GTT TGA TCM TGG CTC AG - 3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T - 3′) were used. The polymerase chain reaction mixture (30 μL) include the following: 3 μL of 10×reaction buffer, 1 μL of 10 mM dNTPs, 1 μL of 5×Band doctor, 1 μL of each 10 pmol primer, 1 μL template DNA, 21.5 μL sterilized distilled water, and 0.2 μL of 2.5 U EF-Taq polymerase (Solgent, Daejeon, Korea). Activation of

Taq polymerase at 95°C for 2 min, 35 cycles of 95°C for 1 min, 55°C, and 72°C for 1 min each were performed, finishing with a 10 min step at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA). The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, USA). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, USA).

Initial contamination level: The prevalence data was fitted to beta distribution (α_1; the number of positive samples+1, α_2; tested total samples-positive samples+1), and contamination levels during storage at cheese factories were fitted to a uniform distribution (a; minimum contamination level, b; maximum contamination level) (Vose, 1998). Eventually, initial contamination level (Log CFU/g) in the risk assessment model was calculated by prevalence×contamination level.

Estimating contamination levels at consumption: To identify contamination levels of cheese at retail, natural and processed cheese were purchased at a local supermarket and 25 g (natural) or 1 slice (processed) of cheese samples were aseptically placed into a sample bag (3M, USA). The analysis was performed using the same method described in section of prevalence.

Because *C. perfringens* usually exists in spore form in cheese, the survival of *C. perfringens* spore on cheese was evaluated at 4°C, 25°C, and 30°C. *C. perfringens* strains NCCP10846 and NCCP10970 were incubated in cooked meat medium (Oxoid Ltd., England) at 37°C for 24 h in anaerobic condition under gas pack (AnaeroGen, Oxoid, England) packaging. An aliquot (0.1 mL) of the culture was transferred into 10 mL of fluid thioglycollate (FTG; Difco, USA) broth and heated at 75°C for 20 min. The *C. perfringens* spore cultures were incubated at 37°C for 18 h, and the cultures were subsequently transferred into 10 mL of fresh FTG broth and incubated at 37°C for 4 h. These incubated cultures were added to a modified Duncan and Strong medium (proteose pepton 15.0 g, yeast extract 4.0 g, sodium thioglycolate 1.0 g, sodium phosphate 10.0 g, raffinose 4.0 g, 0.51 mM caffein 50.0 mL in 1 L of distilled water) to obtain 5% concentration of the culture and incubated in an anaerobic jar at 37°C for 48 h. The spores were harvested by centrifugation and washed twice with phosphate buffered saline (pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4, 8.0 g of NaCl, and 0.2 g of KCl in 1 L of distilled water). One-tenth milliliter of spore mixture containing two *C. perfringens* strains was inoculated on natural cheeses (Brie and Camembert cheeses; 15 g) and the cheeses were

placed into a plastic bag to simulate commercial packaging, followed by storage at 4°C, 25°C, and 30°C for 720, 144, and 96 h, respectively. Brie and Camembert cheese were chosen for this experiment because higher moisture content of Brie and Camembert cheese than other cheese allow bacterial growth. Bacterial populations of *C. perfringens* were enumerated on TSC agar overlayed with egg yolk free TSC agar at 37°C for 24 h. However, there was no growth of *C.*

perfringens even on both cheeses at 4°C, 25°C, and 30°C on the Brie and Camembert cheeses (Figure 2). Thus, initial concentration was considered as the final concentration at the point of consumption under cheese storage conditions.

Temperature during storage, retail display, transport, and home storage: To simulate cheese storage, the temperature profile at retail display and in home refrigerators were prepared from studies by MFDS (2007) and Park
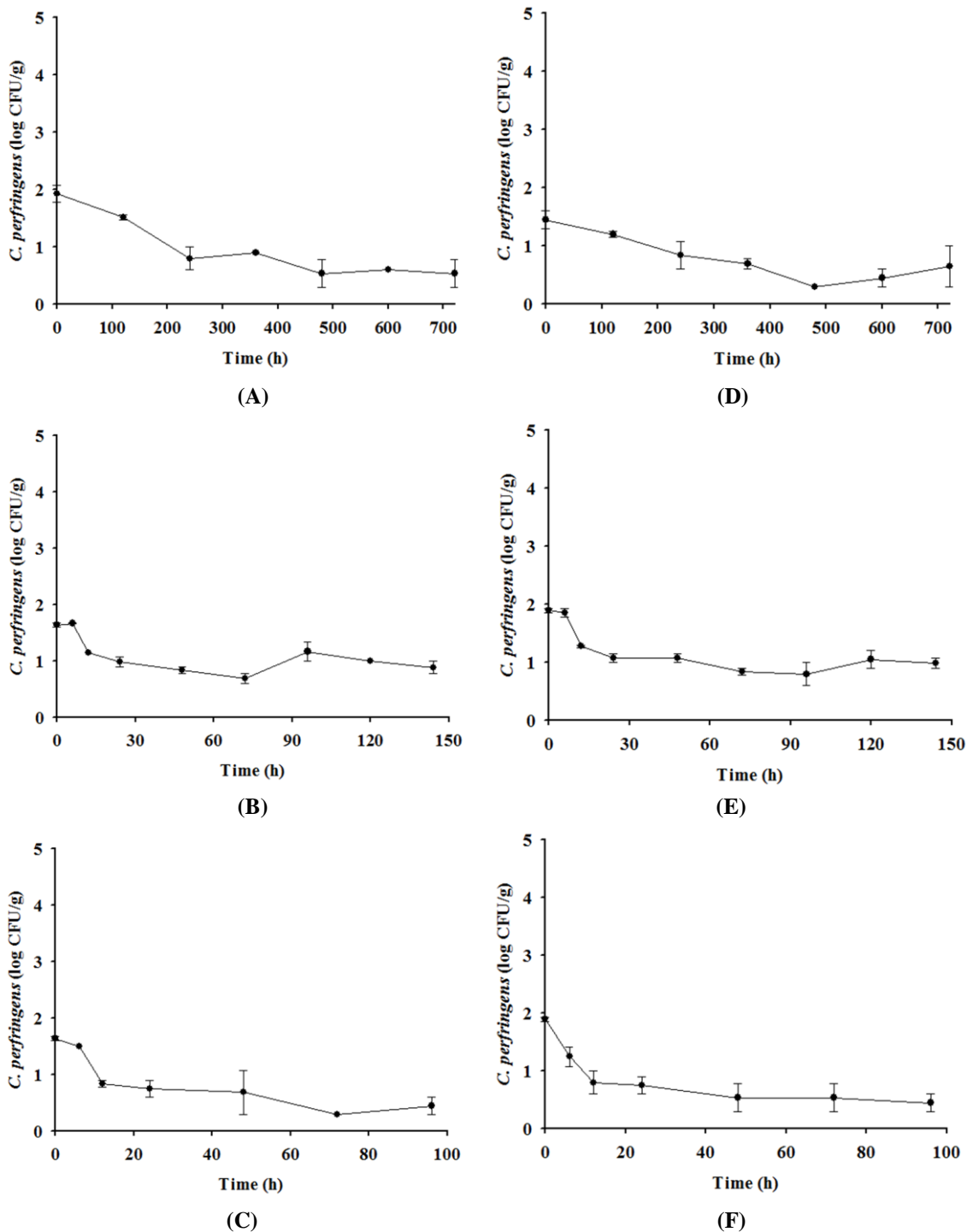

Figure 2. Bacterial populations of *Clostridium perfringens* in Brie (A-C) and Camembert (D-F) cheese during storage at 4°C (A, D), 25°C (B, E), 30°C (C, F) for 720, 144, and 96 h, respectively.

(2010). In Korea, the mean of the temperature of cold-chain food was 7°C, and maximum and minimum temperatures were 22.9°C and –0.4°C, respectively (MFDS, 2007). In addition, a study by Park (2010) showed that the temperature of home refrigerators was 3.5°C±3.0°C.

Consumption of cheese in Korea: To estimate the amounts of natural and processed cheese consumption, raw data were extracted from the Korea National Health and Nutrition Examination Survey 2011 (KNHNES) using the frequency procedure (proc freq) of the SAS program version 9.1 (SAS Institute Inc., Cary, NC, USA). Each data was then fitted to @RISK program version 5.7 (Palisade Corp., Ithaca, NY, USA) to determine appropriate data distributions. The consumption frequencies for natural and processed cheese were estimated by calculating the number of total respondents divided by the number of people who ingested natural and processed cheeses, respectively.

Hazard characterization (dose-response model)

To estimate the probability of foodborne illness from the consumption of *C. perfringens* cells, the following dose-response model for *C. perfringens* developed by Golden et al. (2009) was used;

$$P = 1-\exp(r \times N) \ (r = 1.82 \times 10^{-11})$$

where P is probability of illness, r is the probability of *C. perfringens* single cells causing foodborne illness and N is the cell number ingested during *C. perfringens* consumption

(CFU/serving).

Risk characterization

With exposure assessment data which included prevalence, contamination level, storage temperature distribution, cheese consumption amount and frequency, and dose-response model, the probability of illness per person per day was estimated by simulation using the @RISK program with settings for 10,000 iterations of Latin Hypercube sampling (Tables 1 and 2).

RESULTS AND DISCUSSION

Exposure assessment

Initial contamination level of C. perfringens: The contamination level of *C. perfringens* on cheese was investigated in two cheese factories for each manufacturing step from raw milk to packaged cheese as well as in grocery stores in five different cities through the country. In addition, samples were collected in summer and winter to investigate the prevalence of *C. perfringens* because environmental temperature and humidity may influence contamination levels of the pathogen. For all cheese samples, *C. perfringens* was below the detection limit (natural cheese: 0.30 Log CFU/g; processed cheese: 0.45 Log CFU/g), especially for the packaged cheese from the cheese factories, which was considered the step for initial contamination level in QMRA. Since the contamination level of *C. perfringens* was below detection level in all samples, the data were fitted with the

Table 1. Simulation model and formulas in Excel spreadsheet used to calculate the risk of *Clostridium perfringens* in natural cheese with @RISK

Input model	Unit	Variable	Formula	References
Product				
Pathogens contamination level				
C. perfringens prevalence		PR	= RiskBeta(1,91)	Vose (1998)
Concentration	CFU/g	C	= RiskUniform(0,2)	
Initial contamination level	CFU/g	IC	= PR×C	
	log CFU/g	log(IC)	= log(PR×C)	
Consumption				
Daily consumption average amount	g	Consump	= RiskPearson5[2.6488,25.81,RiskTruncate(0,100), RiskShift(–3.2572)]	KNHNES[a]
Daily consumption frequency	%	ConFre	Fixed 3.894	KNHNES[a]
		CF(0)	= 1–3.894/100	KNHNES[a]
		CF(1)	= 3.894/100	KNHNES[a]
		CF	= RiskDiscrete{[0,1],[CF(0),CF(1)]}	KNHNES[a]
		ConFre	= IF(CF = 0,0,Consump)	KNHNES[a]
Dose-response				
C. perfringens amount		D	= $10^{\log(IC)}$ ×ConFre	
Parameter of r		r	= Fixed 1.82×10⁻¹¹	Golden et al. (2009)
Risk				
Probability of illness/person/d		Risk	= 1–exp(–r×D)	Golden et al. (2009)

[a] 2011 Korea National Health and Nutrition Examination Survey.

Table 2. Simulation model and formulas in Excel spreadsheet used to calculate the risk of *Clostridium perfringens* in processed cheese with @RISK

Input model	Unit	Variable	Formula	References
Product				
Pathogens contamination level				
C. perfringens prevalence		PR	= RiskBeta(1,309)	Vose (1998)
Concentration	CFU/g	C	= RiskUniform(0,2.8)	
Initial contamination level	CFU/g	IC	= PR×C	
	log CFU/g	log(IC)	= log(PR×C)	
Consumption				
Daily consumption average amount	g	Consump	= RiskWeibull[1.3482,20.932,RiskShift(0.26384), RiskTruncate(0,100)]	KNHNES[a]
Daily consumption frequency	%	ConFre	Fixed 2.323	KNHNES[a]
		CF(0)	= 1–2.323/100	KNHNES[a]
		CF(1)	= 2.323/100	KNHNES[a]
		CF	= RiskDiscrete{[0,1],[CF(0),CF(1)]}	KNHNES[a]
		ConFre	= IF(CF = 0,0,Consump)	KNHNES[a]
Dose-response				
C. perfringens amount		D	= $10^{\log(IC)}$ ×ConFre	
Parameter of r		r	= Fixed 1.82×10^{-11}	Golden et al. (2009)
RISK				
Probability of illness/person/d		Risk	= 1–exp(–r×D)	Golden et al. (2009)

[a] 2011 Korea National Health and Nutrition Examination Survey.

beta distribution ($\alpha_1 = 1$, $\alpha_2 = 91$ and $\alpha_1 = 1$, $\alpha_2 = 309$ for natural and processed cheese) and the uniform distribution (a = 0, b = 2, and a = 0, b = 2.8 for natural and processed cheese) for prevalence and *C. perfringens* concentration, respectively. The initial contamination levels were then calculated by prevalence× concentrations of *C. perfringens*, and the initial contamination levels were –2.35 log CFU/g and –2.73 log CFU/g for natural and processed cheese, respectively. Beta distribution is the result of applying Bayes' Theorem to the observed events, and uniform distribution can be used for the case with lack of information for the probability of event prior to the observed events (Vose, 1998).

C. perfringens growth during distribution, display for sale and consumption: In the exposure assessment, since *C. perfringens* did not grow even at 30°C for 96 h (Figure 2) and *C. perfringens* spores persist in environments in which they germinate, followed by rapid growth at optimum temperature (43°C to 47°C) (Le Loir, 2003; CDC, 2014), the pathogen may not grow at the temperature range for distribution, display at grocery stores, and storage at home. The storage conditions at retail and home did not affect *C. perfringens* growth because the storage temperature at retail locations and at home was too low for *C. perfringens* growth. Although a presumptive colony was found in one sample, 16s DNA sequencing result showed that the colony was not *C. perfringens*. Hence, it was assumed that initial contamination level at factories is the same as the contamination level at consumption.

Cheese consumption in Korea: Raw data from the 2011 Korean National Health and Nutrition Examination Survey

(KNHANES) were analyzed with 'Proc freq' of SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) to estimate the daily consumption amount of natural and processed cheese, and the frequency of consumption. Pearson 5 distribution fitting of the raw data showed that the mean consumption amount of natural cheese was 12.40±19.43 g/d (95% confidence interval: 0.915 to 34.90 g/d) (Figure 3A). Regarding processed cheese, the Weibull distribution was appropriate to describe the processed cheese consumption data, and the mean consumption amount of processed cheese was 19.46±14.39 g/d (95% confidence interval: 2.6 to 40.0 g/d) (Figure 3B). Because the frequency of cheese consumption was not available, the ratios (natural cheese: 0.0389; processed cheese: 0.0232) of total respondents (7,704 people) to the respondents (300 people) who consume natural cheese as well as the respondents (179 people) who consume processed cheese were used for the frequency of cheese consumption. The discrete distribution was then fitted to the frequency of consumption (Tables 1 and 2).

Hazard characterizaiton (dose-response model)

The ingested dose of *C. perfringens* was translated into the probability of foodborne illness using the dose-response model developed by Golden et al. (2009), and the dose response model estimated the probability of *C. perfringens* foodborne illness resulting from a certain level of exposure.

Risk characterization

The primary measure of risk used in this assessment was the risk of *C. perfringens* per serving of cheese, which is
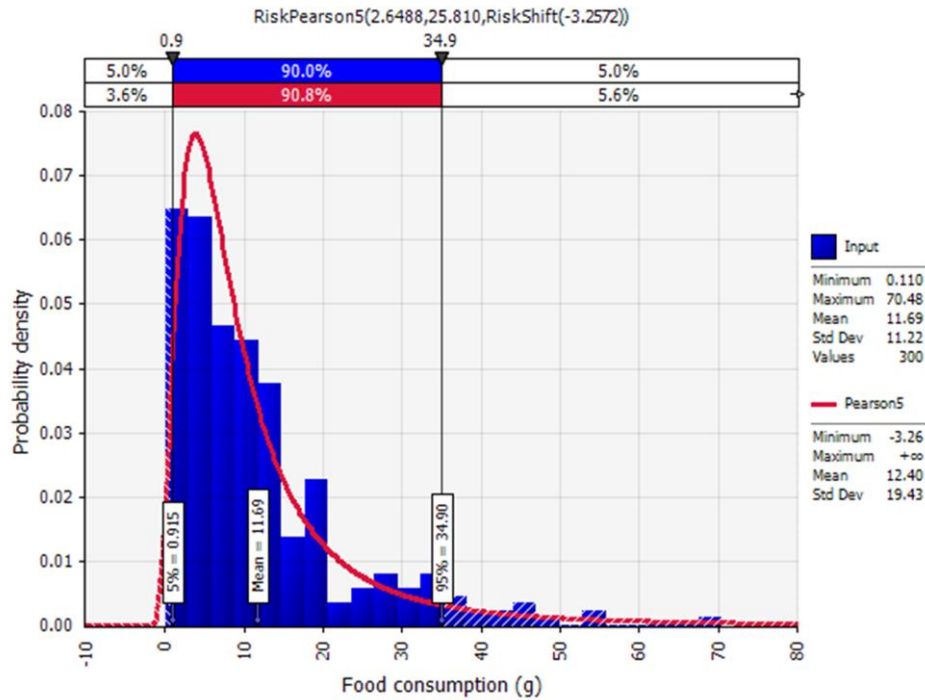
(A)
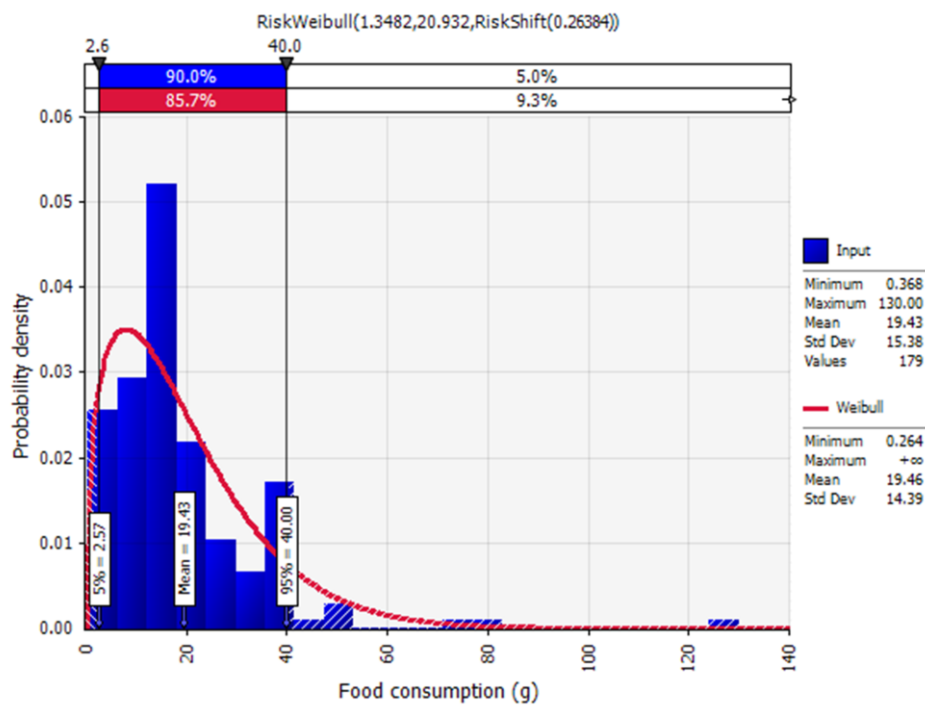
(B)

Figure 3. Probabilistic distribution for daily intake of natural (A) and processed (B) cheeses in the Korea National Health and Nutrition Examination Survey (KNHNES) in 2011.

useful in comparing risk between different cheese types. The concept of risk also contains the severity of the consequences of exposure. Hence, the consumption amounts of natural and processed cheeses were considered to estimate public health risk.

In the model, 10,000 iterations were conducted for each category of products. Within each simulation, the values of other input variables were randomly sampled from their corresponding distribution with a total of 10,000 iterations. The estimated probability of *C. perfringens* foodborne illness

Table 3. Probability of foodborne illness from *Clostridium perfringens* per person per day with the consumption of natural and processed cheeses

Probability illness/person/d	5%	25%	50%	95%	99%	Maximum	Mean
Natural cheese	0	0	0	0	2.46×10^{-12}	6.64×10^{-11}	9.57×10^{-14}
Processed cheese	0	0	0	0	8.94×10^{-13}	1.82×10^{-11}	3.58×10^{-14}

for natural cheese and processed cheese are presented in Table 3. These results were derived from data, assumption and knowledge summarized in exposure assessment and hazard characterization. The estimated probabilities of *C. perfringens* foodborne illness per person were 9.57×10^{-14} and 3.58×10^{-14} for natural cheese and processed cheese, respectively, and even the 99th percentile was 2.64×10^{-12} and 8.94×10^{-13} for natural cheese and processed cheese, respectively (Table 3). Although processed cheese had a higher annual consumption amount per person compared to natural cheese, the annual risk estimate of processed cheese was lower than that of natural cheese, which may be due to the lower frequency of consumption for the cheese. According to the estimates, the annual *C. perfringens* foodborne illness related to cheese in Korea was 1.75×10^{-3} and 6.53×10^{-4} for natural cheese and processed cheese, which means the incidence of the foodborne illness was 3.49×10^{-5} and 1.31×10^{-5} per million people/year, respectively. This calculation method for the annual incidence of the foodborne illness per million people (the annual incidence of the foodborne illness per person×1 million people) is based on independent events, which means that a million people have the same possibility (susceptibility) to be infected with *C. perfringens*. In a study by Ding et al. (2013), the probabilities of listeriosis illness per person per day from eating lettuce at a restaurant and at home in Korea were 3.64×10^{-9} to 1.30×10^{-7}, and 4.71×10^{-8} to 1.68×10^{-6}, respectively. In addition, the probability of *C. perfringens* foodborne illness from the consumption of ham and sausage per person per day in Korea was $3.97\times10^{-11}\pm1.80\times10^{-9}$ (Ko et al., 2012). The values were higher compared to the probability of *C. perfringens* foodborne illness by cheese consumption, indicating that the risk of *C. perfringens* foodborne illness from cheese consumption can be considered relatively low. The International Commission on Microbiological Specification for Foods also considers the risk of *C. perfringens* is moderate (Forsythe, 2002; FDA, 2012). In reviewing *C. perfringens*, 58 outbreaks in Korea affected 1,308 patients from 2010 to 2013 (MFDS, 2014b). Among the cases, none were related to cheese consumption. This may be related to the method of cheese consumption pattern in Korea. Koreans usually consume cheese in a melted form, which may destroy vegetative *C. perfringens* cells. The amount of cheese consumption of cheese is also low compared to other countries. These factors may result in low risk of *C. perfringens* in cheese.

In conclusion, a stochastic risk assessment model developed in our study should be useful in QMRA for *C. perfringens* in cheese, and the results indicate that the risk of *C. perfringens* in cheese is low. Therefore, the microbial criteria (n = 5, c = 0, m = 0 for 25 g) for *C. perfringens* in cheese need to be reconsidered.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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