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Original Article

Survival of enteroaggregative *Escherichia coli* and *Vibrio cholerae* in frozen and chilled foods

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Abstract

Introduction: Enteroaggregative *Escherichia coli* (EAEC) and *Vibrio cholerae* are common bacteria that infect people in developing countries. Nowadays, food preservation by freezing and refrigeration are very common practices that extend the shelf life of food products. Unfortunately, EAEC and *V. cholerae* are suspected to survive at low temperatures.

Methodology: Various foods and beverages used as samples were artificially inoculated with EAEC and *V. cholerae* pure colonies and incubated at room temperature (27°C), refrigeration temperature (4°C), and frozen temperature (-20°C) for one week, two weeks, one month, two months, and three months. The survival of these bacteria was confirmed by polymerase chain reaction (PCR) with specific primers to detect their virulence genes (*aggR* for EAEC and *toxR* for *V. cholerae*) that represent the presence of these bacteria.

Results: This study showed that EAEC was able to survive in all food samples used in this study for at least three months under room temperature, refrigeration temperature, and frozen temperature. *V. cholerae* had poor survival in almost all food samples over two months under room temperature and over three months under refrigeration temperature. *V. cholerae* was able to survive in all food samples for at least three months under frozen temperature.

Conclusions: The survival of EAEC and *V. cholerae* in this study should increase our awareness of how hazardous these bacteria are. Therefore, in order to improve food safety and hygiene, advanced preservation and sterilization methods should be discovered to prevent contamination with and survival of EAEC and *V. cholerae*.

Key words: enteroaggregative *Escherichia coli*; *Vibrio cholerae*; artificial contamination; chilled foods; frozen foods.

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Introduction

Escherichia coli is a common bacteria in the intestinal tract of humans and a variety of animals. Most *E. coli* do not cause gastrointestinal illnesses; however, some of *E. coli* strains are pathogenic, including enteroaggregative *E. coli* (EAEC), and cause diarrheal disease. Diarrhea can cause death in those who have experienced dehydration, malnutrition, development deficiencies, and other complications. EAEC is commonly found in people, especially infants and children, in developing countries, including Indonesia. EAEC likely has the ability to evade the immune system and cause persistent infection because it has various gene profiles that encode pathogenic mechanisms [1,2].

Vibrio cholerae is known to be a pathogenic bacteria that mainly lives in sea water [3]. The pathogenicity of *V. cholerae* is due to the expression of the *toxR* gene as a positive transcriptional virulence regulator gene that induces the secretion of enterotoxins [4]. *V. cholerae* can cause cholera by infecting the mucosal epithelium, which often happens in developing countries around the world. Cholera can cause death from dehydration. The infectious dose that can cause severe cholera is about 10^8 bacteria [5].

The number foodborne and waterborne illnesses is increasing. *E. coli* and *V. cholerae* have a high potential as causative agents of foodborne and waterborne illnesses because they can spread through water, uncooked meat and poultry, and untreated sewage [6,7]. Nowadays, food preservation is a common process to prevent food from spoiling, and to prevent the growth of pathogenic bacteria and molds. There are a number of methods to preserve food, including pasteurization, drying, refrigeration, and freezing. Food preservation has become very important in the food industry. Unfortunately, pathogenic bacteria, including EAEC and *V. cholerae*, are suspected to survive many methods of food preservation [8].

Studies of the survival of EAEC and *V. cholerae* are few, compared to studies on the survival of

enterohemorrhagic *Escherichia coli* O157:H7. Therefore, it is necessary to conduct research to examine the survival of EAEC and *V. cholerae* in frozen and chilled foods.

The aim of this work was to study the survival of EAEC and *V. cholerae* in some artificially infected frozen and chilled foods kept under different temperatures for different periods. The study of the viability of these pathogens under the same conditions was another goal of this work.

Methodology

Culture preparation

The pathogenic bacteria used in this study were EAEC provided by the United States (US) Naval Medical Research Unit No. 2 and *V. cholerae* (B.NJ.1.1, *tox*R$^+$) originally isolated from edible ice [9]. Both strains were activated by streaking on nutrient agar (NA) (Oxoid, Basingstoke, England) and were then incubated at 37°C overnight.

Sample preparation

Samples of frozen and chilled foods including nuggets, fish meatballs, fresh chicken meat, frozen peas, frozen corns, pudding, chocolate ice cream, carrot juice, ultra-high temperature processing (UHT) milk, and drinking water were used in this study. Food samples of 5 g were inserted into sterile bottles. Food samples were homogenized with a blender to mix with the culture. There was a total of 60 bottles (three different incubation temperatures, five different durations of incubation, two different pathogenic bacteria, and two replications).

Inoculation of cultures to samples

EAEC colonies from NA were subcultured in EC broth media (Oxoid) and incubated in 37°C 120 rpm for about 18 hours to get the log phase. Meanwhile, *V. cholerae* colonies from NA were subcultured in alkaline peptone water (APW) media (Oxoid) and incubated in 37°C 120 rpm for about 18 hours to get the log phase. After 18 hours, the culture was homogenized to ensure the culture suspension. The concentration of cells was measured using a spectrophotometer (Thermo Scientific Genesys 20, Waltham, USA) at 600 nm. To equalize the concentration of cells in every inoculation to the sample, EAEC culture was diluted to the equivalent of a McFarland standard 0.5 (% transmittance = 74.3, absorbance at 600 nm = 0.132). McFarland standard 0.5 equals 1.5×10^8 cell count density.

Food samples were inoculated with 1 mL of diluted bacteria. Then samples were incubated at room temperature (27°C), refrigeration temperature (4°C), and frozen temperature (-20°C). The food samples were analyzed after one week, two weeks, one month, two months, and three months.

Sample preparation before detection

After being incubated, EAEC and *V. cholerae* were detected by polymerase chain reaction (PCR). Inoculated food samples were added to 15 mL physiological solution 0.85% (w/v) (Oxoid). One milliliter of each sample was diluted into 5 mL EC broth for EAEC and 5 mL of APW media for *V. cholera*, and then incubated overnight in 37°C 120 rpm. One loopful of suspension in EC broth was streaked in eosin methylene blue (EMB) agar (Oxoid), which is a selective media, to check the viability of EAEC. Meanwhile, one loopful of suspension in APW media was also streaked in thiosulfate citrate bilesalts sucrose (TCBS) (Oxoid), which is also a selective media, to check the viability of *V. cholerae*. Then, EMB and TCBS agars were incubated at 37°C overnight. Positive *E. coli* colonies were metallic green sheen colonies in EMB, while positive *V. cholerae* colonies were yellow colonies in TCBS. These validations in EMB and TCBS were done after every incubation time.

PCR detection

The detection of EAEC was focused on the *aggR* gene, while *V. cholerae* was focused on the *toxR* gene. One milliliter of the suspension was taken and centrifuged at 8,000 rpm for 2 minutes. Then, pellets were re-suspended by 200 µL nuclease-free water and boiled in boiled water for about 15 minutes. After boiling, the suspension was centrifuged at 12,000 rpm 4°C for 2 minutes. The supernatant was used as a DNA template.

Table 1. Sequence of primers used for detection of virulence genes in this study 5'→3'

Gene	Primer name	Primer sequences (5' → 3')	Amplicon size (bp)	Annealing temp (°C)	Reference
aggR	*aggR*-Fwd	GTATACACAAAAGAAGGAAGC	254	52	[10]
	aggR-Rvs	ACAGAATCGTCAGCATCAGC			
toxR	*toxR*-Fwd	CCTTCGATCCCCTAAGCAATA	779	62	[11]
	toxR-Rvs	AGGGTTAGCAACGATGCGTAAG			

PCR mixture consisted of 12.5 µL GoTaq Green Master Mix (Promega, Fitchburg, USA); 1 µL of each of primer, forward and reverse (Eurogentec-AIT Biotech, Singapore, Singapore) as listed in Table 1 [10,11]; 2 µL of DNA template; and 8.5 µL nuclease-free water. PCR was performed in a thermal cycler, Bio-Rad C-1000 (Biorad, Hercules, USA). The PCR running condition for *aggR* was as follows: pre-denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 1 minute, annealing at 52°C for 1 minute, elongation at 72°C for 1 minute, followed by post-elongation at 72°C for 10 minutes [10]. Meanwhile, the PCR running condition for *toxR* was as follows: pre-denaturation at 94°C for 2 minutes, 25 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, elongation at 72°C for 1 minute, followed by post-elongation at 72°C for 10 minutes [12].

After PCR was performed, the DNA was visualized with electrophoresis using 1.8% agarose and 1 kb DNA ladder (Geneaid, New Taipei City, Taiwan) as a marker. Positive controls were EAEC and *V. cholerae* (B.NJ.1.1, *tox*R^{+}) pure colonies. The electrophoresis running condition was 100 voltage for 45 minutes using TAE 1X buffer.

Sequencing of aggR and toxR genes

The amplified genes were sequenced to confirm that they were the *aggR* gene from EAEC and the *toxR* gene from *V. cholerae*. About 100 µL of unpurified amplified genes from random food samples was sequenced. The sequencing phase was done in PT Genetika Science Indonesia, Jakarta. The FASTA sequence was analyzed with nucleotide BLAST from the National Center for Biotechnology Information (NCBI) (http://blast.ncbi. nlm.nih.gov/Blast.cgi).

Results

PCR detection

The virulence genes of EAEC and *V. cholerae* were amplified using PCR with specific primers. *aggR* gene detection after various incubation durations is presented in Table 2 and Figure 1. The *aggR* genes were detected in all food samples after one week up to

Table 2. *aggR* gene detection in frozen and chilled foods after various incubation durations

Samples	1 Week			2 Weeks			1 Month			2 Months			3 Months		
	R	K	F	R	K	F	R	K	F	R	K	F	R	K	F
Nugget	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fish meatball	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fresh chicken meat	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pea	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Corn	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pudding	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ice cream	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carrot juice	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
UHT milk	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Drinking water	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

UHT: ultra-high temperature processing; R: room temperature (27°C); K: refrigeration temperature (4°C); F: frozen temperature (-20°C); + Presence of *aggR* gene; - absence of *aggR* gene.

Table 3. *toxR* gene detection in chilled and frozen foods after various incubation durations

Samples	1 Week			2 Week			1 Month			2 Month			3 Months		
	R	K	F	R	K	F	R	K	F	R	K	F	R	K	F
Nugget	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Fish meatball	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Fresh chicken meat	-	+	+	-	+	+	-	+	+	-	+	+	-	-	+
Pea	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
Corn	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Pudding	+	+	+	+	+	+	-	+	+	-	+	+	-	-	+
Ice cream	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Carrot juice	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
UHT milk	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Drinking water	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

UHT: ultra-high temperature processing; R: room temperature (27°C); K: refrigeration temperature (4°C); F: frozen temperature (-20°C); + Presence of *toxR* gene; - absence of *toxR* gene.

three months at room temperature (27°C), refrigeration temperature (4°C), and frozen temperature (-20°C). These results indicated that EAEC can survive in all foods for at least three months.

toxR gene detection after various incubation durations is presented in Table 3 and Figure 2. At room temperature (27°C), the *toxR* gene was detected in all samples up to three months, except in fresh chicken meat, pudding, nuggets, fish meatballs, frozen peas, and frozen corn. The *toxR* gene was not detected in fresh chicken meat after one week of incubation, while in pudding after one month of incubation. In frozen peas, the *toxR* gene was not detected after two months of incubation. Meanwhile, in nuggets, fish meatballs, corn, and chocolate ice cream the, *toxR* gene was not detected after three months of incubation.

At refrigeration temperature (4°C), the *toxR* gene was detected in all samples up to three months, except in fresh chicken meat, nuggets, fish meatballs, frozen peas, and pudding. The *toxR* gene was not detected in fresh chicken meat after one month of incubation, while in nuggets, fish meatballs, frozen peas, and pudding after three months of incubation.

Under frozen temperature (-20°C), the *toxR* gene was detected in all samples after one week up to three months. These results indicated that *V. cholerae* had the longest survival time in all foods under frozen temperature for at least three months.

Sequencing of aggR and toxR genes

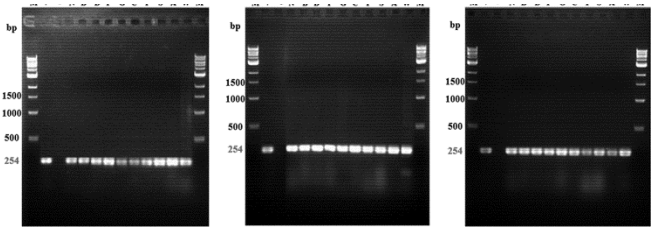
The *aggR* gene and *toxR* gene, which were detected as target genes to confirm the survival of EAEC and *V. cholerae*, were sent for sequencing by PT Genetika Science Indonesia, Jakarta. The FASTA sequence analysis by nucleotide BLAST from NCBI showed 100% similarity with the *aggR* gene of EAEC and 99% similarity with the *toxR* gene of *V. cholerae*.

Discussion

By artificially contaminating food and beverage samples with EAEC, the authors discovered and confirmed with two replications that EAEC was able to survive under three different temperatures in various samples for at least three months, which is in agreement with the findings of a previous study reporting the ability of EAEC to survive in response to nutrient limitation and adverse environmental conditions that persist in the prolonged stationary phase [13,14].

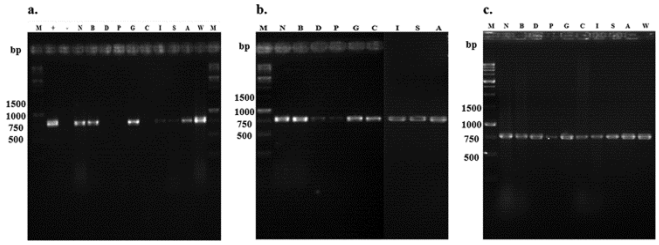
The survival of *V. cholerae* under three different temperatures in various samples varied among

Figure 1. *aggR* gene amplification using PCR after two months of incubation under (a) room temperature (27^{o}C), (b) refrigeration temperature (4^{o}C), (c) frozen temperature (-20^{o}C).



Marker (M): 1 kb DNA ladder (Geneaid, Taiwan); (+): positive control (EAEC pure colonies); (-): negative control; (N): nuggets; (B): fish meatballs; (D): fresh chicken meat; (P): peas; (G): corn; (C): pudding; (I): chocolate ice cream; (S): UHT milk; (A): drinking water; (W): carrot juice

Figure 2. *toxR* gene amplification using PCR after two months of incubation under (a) room temperature (27^{o}C), (b) refrigeration temperature (4^{o}C), (c) frozen temperature (-20^{o}C).



Marker (M): 1 kb DNA ladder (Geneaid, Taiwan); (+): positive control (*V. cholerae* [B.NJ.1.1, *tox*R^{+}] pure colonies); (-): negative control; (N): nuggets; (B): fish meatballs; (D): fresh chicken meat; (P): pea; (G): corn; (C): pudding; (I): chocolate ice cream; (S): UHT milk; (A): drinking water; (W): carrot juice

samples. These results also showed similarity with the findings of previous studies. At room temperature, authors of previous studies reported that *V. cholerae* was able to survive in milk and water for the longest period, followed by ice cream and corn. The shortest survival period was in fish meatballs, peas, and pudding. In ice cream and corn, *V. cholerae* had almost the same length of survival [15,16].

Under refrigeration temperature (4°C), the survival of *V. cholerae* was also reported previously [15,16]. It showed the same pattern that was found in this study. *V. cholerae* was able to survive in ice cream, milk, and water for the longest period, followed by peas and pudding. The shortest survival period was in fish meatballs. In peas and pudding, *V. cholerae* had almost the same period of survival. Moreover, the survival period of *V. cholerae* in corn in this study was better than that reported in the previous studies [15,16].

Unfortunately, the survival of *V. cholerae* in nuggets and fresh chicken meat was not measured and

analyzed in previous studies, whether under room temperature (27°C), refrigeration temperature (4°C), or frozen temperature (-20°C). The differences in *V. cholerae*'s survival period among these studies were due to the differences in food composition and characteristics, number of inoculums, secondary flora or endogenous microorganisms in foods, and also the handling and processing techniques used on the foods [15,16].

The samples used in this study were very diverse, including raw foods, cooked foods, high-acidity drinks, drinking water, vegetables, and high-sugar foods. Moreover, food and beverage samples were not sterilized before EAEC pure culture and *V. cholerae* pure culture were inoculated into the samples. This will increase the competition for the EAEC and *V. cholerae* to survive, especially under incubation in room temperature (±27°C) which allowed other fast-growing microorganisms to grow by utilizing nutrients in foods [17]. In addition, the minimum growth temperature for *V. cholerae* is 15°C, while for EAEC is 7–8°C [18,19]. This competition inhibited the survival of *V. cholerae* at room temperature after one week of incubation in fresh chicken meat.

After several days of incubation time, the environmental conditions tend to be adverse, including limited nutrition, high acidity (acid stress), osmotic shock, and limited oxygen level. These adverse conditions should have inhibited the growth of microorganisms, including EAEC, but in fact EAEC was able to survive. The survival of EAEC was also supported by its capability to keep growing under aerobic and anaerobic conditions [20]. EAEC utilizes glucose and produces acetate as a major fermentative product under aerobic conditions, then uses acetate as a carbon source during the early stationary phase of growth. When acetate is used up, amino acids will be used as carbon and nitrogen sources during the stationary phase of growth [14].

EAEC survival also occurred because EAEC has the *rpoS* gene, which is a regulator gene. This gene is an alternate sigma factor (σ) of RNA polymerase, which plays an important role in the survival of several foodborne human pathogens. The gene *rpoS* is involved in stress resistance and protection under adverse environmental conditions such as acid stress, nutrient limitation, and osmotic shock [21]. Moreover, the survival of EAEC is also related to the growth phase. The stationary phase makes EAEC more resistant to acid than it is in the exponential phase. Once induced, the *rpoS* gene will remain active until cell growth reenters the log phase or a normal situation. RpoS will be rapidly degraded by ClpXP proteases [21,14].

E. coli, including EAEC, also has Fis (factor for inversion stimulation), which is a nucleotide-associated protein. Fis levels peak during the early growth phase and become very low during the stationary phase. Fis plays a role in regulating efficient nutrient uptake and rapid growth. Therefore, during poor nutritional conditions, Fis levels tend to be very low to slow down the growth of EAEC by regulating nutrient uptake as efficiently as possible [14]. Moreover, during the adverse nutritional condition, including the stationary phase and long-term stationary phase, Fis upregulates genes for cell processes, including genes for adaptation and protection [22].

During the stationary phase, nutrients become exhausted, while waste products accumulate. This condition causes a stress to the cells and leads to the death phase. During this phase, the cells become viable but non-culturable (VBNC), and nutrients from these kind of cells will be released into the environment, where they will be used by the remaining viable and culturable cells. Damaged cells in the stationary phase will be lysed by σ^E. This σ^E will reduce outer membrane proteins (OMP) in the outer membrane, which will be followed by disintegration of the outer membrane [23,14]. The ability to enter a VBNC state occurs not only in EAEC, but also in *V. cholerae*. Lack of ability to enter a VBNC state causes damage to the cell membrane due to the formation of ice crystals at frozen temperature, which can further reduce the competition in the population [24,25].

V. cholerae also have the ability to form cold shock proteins (Csp) in the VBNC state. These proteins act as nucleic acid chaperones to repair several enzymes folding that are damaged during low temperature, thus enabling the enzymes to work normally. These proteins can also stabilize RNA secondary structures, induce negative supercoiled DNA, and bind to nucleic acids to regulate the replication, transcription, and translation [24,26]. CspV is the only true Csp that is induced in *V. cholerae* at low temperatures. CspV is stable and highly expressed at low temperatures [27].

In addition, the survival of bacteria in foods under low temperature also depends on nutrition composition. Food samples with abundant nutrition composition, such as nuggets, fish meatballs, fresh chicken meat, and peas allow almost all microorganisms to grow, which actually causes tighter competition [28]. High level of high molecular weight (HMW) organic compounds, such as fibers, can inhibit

bacterial enzyme activities by forming complexes with the enzymes [29]. In this study, high levels of HMW organic compounds were found in pudding and peas.

The presence of colloids and sugars also influences the survival of EAEC and *V. cholerae*. The rate of crystallization is also decreased in the presence of solutes or colloid, such as milk. Colloids will absorb some of the free water to keep the ice crystals small, hence protecting the cells from lysis. Meanwhile, the presence of sugars also helps to stabilize the osmotic pressure on the cell during the freezing and thawing process [25].

Conclusions

EAEC is one of the pathogenic strains of *E. coli*. EAEC can survive in all foods for at least three months under frozen temperature (-20°C), refrigeration temperature (4°C), and room temperature (27°C). Meanwhile, *V. cholerae* can survive in all foods for at least three months under frozen temperature (-20°C) and for two months under refrigeration temperature (4°C), and for one month under room temperature (27°C) in nearly all foods, except in fresh chicken meat and pudding.

The ability to survive under low temperatures indicates that even though foods and beverages are preserved by freezing or are refrigerated, EAEC and *V. cholerae* are still able to survive. Therefore, in order to improve foods safety and hygiene, advanced preservation and sterilization methods should be developed to prevent the survival and the contamination of EAEC, *V. cholerae*, and other pathogenic bacteria in foods.

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Authors' contributions

All authors contributed equally to this work. All authors designed the experiment. SA and YEK performed the experiments and obtained the data. DEW supervised development of the project. All authors analyzed and interpreted the data. SA and YEK wrote the manuscript. DEW helped in manuscript evaluation and acted as corresponding author.

References

1. Huang DB, Mohamed JA, Nataro JP, DuPont HL, Jiang ZD, Okhuysen PC (2007) Virulence characteristics and the molecular epidemiology of enteroaggregative *Escherichia coli* isolates from travellers to developing countries. J Med Microbiol 56: 1386-1392.
2. Okhuysen PC, DuPont HL (2010) Enteroaggregative *Escherichia coli* (EAEC): a cause of acute and persistent diarrhea of worldwide importance. J Infect Dis 202: 503-505.
3. Goel AK, Jain M, Kumar P, Sarguna P, Bai M, Ghosh N, Gopalan N (2011) Molecular characterization reveals involvement of altered El Tor Biotype *Vibrio cholera*e O1 strains in cholera outbreak at Hyderabad, India. J Microbiol 49: 280-284.
4. Pang L, Zhang XH, Zhong Y, Chen J, Li Y, Austin B (2006) Identification of *Vibrio harveyi* using PCR amplification of the *toxR* gene. Appl Microbiol 43: 249-255.
5. Kitaoka M, Miyata ST, Unterweger D, Pukatzki S (2011) Antibiotic resistance mechanisms of *Vibrio cholerae*. J Med Microbiol 60: 397-407.
6. Kaper JB, Nataro JP, Mobley HLT (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol 2: 123-140.
7. Mrityunjoy A, Kaniz F, Fahmida J, Shanzida JS, Aftab U, Rashed N (2013) Prevalence of *Vibrio cholerae* in different food samples in the city of Dhaka, Bangladesh. Int Food Research J 20: 1017-1022.
8. Fellers CR (2005) Public health aspects of food preservation. Am J Public Health 17: 470-475.
9. Waturangi DE, Wennars M, Suhartono MX, Wijaya YF (2013) Edible ice in Jakarta, Indonesia, is contaminated with multidrug-resistant *Vibrio cholerae* with virulence potential. J Med Microbiol 62: 352-359.
10. Toma C, Lu Y, Higa N, Nakasone N, Chinen I, Baschkier A, Rivas M, Iwanaga M (2003) Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. J Clin Microbiol 41: 2669-2671.
11. Bhowmick TS, Das M, Ruppitsch W, Stoeger A, Pietzka AT, Allerberger F, Rodrigues DP, Sarkar BL (2009) Detection of virulence-associated and regulatory protein genes in association with phage typing of human *Vibrio cholera* from several geographical regions of the world. J Med Microbiol 58: 1160-1167.
12. Rivera IN, Chun J, Huq A, Sack RB, Colwell RR (2001) Genotypes associated with virulence in environmental isolates of *Vibrio cholerae.* Appl Microbiol 67: 2421-2429.
13. Družina VB, Butorac A, Mrvčić J, Dragičević TL, Tomas VS (2011) Bacterial stationary-phase evolution. Food Technol Biotechnol 49: 13-23.
14. Shimizu K (2014) Regulation Systems of Bacteria such as *Escherichia coli* in response to nutrient limitation and environmental stresses. Metabolites 4: 1-35.
15. Felsenfeld O (1965) Notes on food, beverages and fomites contaminated with *Vibrio cholerae.* Bull World Health Organ 33: 725-734.
16. International Commission on Microbiological Specifications for Foods (2003) Microorganisms in foods 6: microbial ecology of food commodities. New York: Kluwer Academic/Plenum Pub. 174-249.
17. Goto M (1992) Fundamentals of bacterial plant pathology. California: Academic Press. 342 p.
18. Fisher K, Philips C (2009) The ecology, epidemiology and virulence of *Enterococcus.* J Microbiol 155: 1749-1757.

19. Yates A (2011) Shiga toxin-producing *Escherichia coli* (STEC). In Craig D, Healy M, editors. Food Standards Australia New Zealand - Agents of Foodborne Illness, 2nd edition: A Technical series summarizing key information on microorganisms associated with foodborne illness. Canberra: Food Standards Australia New Zealand (FSANZ). 77-84.
20. Torres AG, Hernández MMPA, Laguna YM (2010) Overview of *Escherichia coli*. In Torres AG, editor. Pathogenic *Escherichia coli* in Latin America. Sharjah: Bentham Science Publishers. 1-7.
21. Kaur P, Chakraborti A (2010) Proteome analysis of a food borne pathogen *Enteroaggregative Escherichia coli* under acid stress. J Proteomics Bioinformatics 3: 10-19.
22. Bradley MD, Beach MB, Koning APJ, Pratt TS, Osuna R (2007) Effect of Fis on *Escherichia coli* gene expression during different growth stages. Microbiology 153: 2292-2940.
23. Nagamitsu H, Murata M, Kosaka T, Kawaguchi J, Mori H, Yamada M (2013) Crucial roles of MicA and RybB as vital factors for σ^E-dependent cell lysis in *Escherichia coli* long-term stationary phase. J Mol Microbiol Biotechnol 23: 227-232.
24. Oliver JD (2005) The viable but nonculturable state in bacteria. J Microbiol 43: 93-100.
25. Woodburn MJ, Strong DH (1959) Survival of *Salmonella typhimurium*, *Staphylococcus aureus*, and *Streptococcus faecalis* frozen in simplified food substrates. Appl Microbiol 8: 109-113.
26. Schmid B, Klumpp J, Raimann E, Loessner MJ, Stephan R, Tasara T (2009) Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. Appl Microbiol 75: 1621-1627.
27. Datta PP, Bhadra RK (2003) Cold shock response and major cold shock proteins of *Vibrio cholerae*. Appl Microbiol 69: 6361-6369.
28. Dave D, Ghaly AE (2011) Meat spoilage mechanisms and preservation techniques: a critical review. Am J Agr Biol Sci 6: 486-510.
29. Kirschner AK, Schlesinger J, Farnleitner AH, Hornek R, Suss B, Golda B, Herzig A, Reitner B (2008) Rapid growth of placktonic *Vibrio cholerae* non-O1/non-O139 strains in a large alkaline lake in Austria: dependence on temperature and dissolved organic carbon quality. Appl Microbiol 74: 2004-2015.

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