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Inhibitory Effects of Acetic Acid and Temperature on Growth of *Campylobacter jejuni* ATCC 33291

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Abstract The growth inhibition of *Campylobacter jejuni* ATCC 33291 in the presence of 1% acetic acid at 4, 25, and 42°C in a medium broth and model system with chicken was investigated. The growth of *C. jejuni* was most sensitively inhibited at 42°C, followed by 25°C and 4°C, at pH 5.5 and pH 6.5, and by the addition of 1% acetic acid. The decimal death rates (D values) of *C. jejuni* with 1% acetic acid at 4, 25, and 42°C were determined to be 22, 8.5, and 1.4 min, respectively, in an FBP-SBB medium. The D values of *C. jejuni* were increased by the addition of chicken and did not follow the linear relationship observed in the FBP-SBB media without chicken. When using distilled water instead of FBP-SBB in the model system, the death rate of *C. jejuni* was dramatically accelerated. The injured or low cell numbers that were impossible to enumerate using the plate count method, were detected by a polymerase chain reaction and enrichment culture procedure. These results suggested that acetic acid is reliable and effective as a disinfectant, however, it is necessary to take additional care at refrigeration temperatures due to the potential of injured cells during poultry processing.

Key words: Inhibitory effect, acetic acid, *Campylobacter jejuni*, D value, PCR

Campylobacter jejuni is an important foodborne pathogen that causes human enteritis, and a great deal of interest has recently been focused on this organism worldwide [2, 4, 9, 24, 25]. The pathogen is commonly found in a wide range of wild and domestic animals and birds [7]. Cross contamination, undercooking, and pasteurization failure have been suggested as factors associated with human disease transmission. To prevent the outbreak of campylobacteriosis, the application of Hazard Analysis Critical Control Point (HACCP) during poultry processing, considered as a major source of *C. jejuni*, would appear to be beneficial. Many previous studies have shown that *C. jejuni* survives better under refrigeration at 4°C than at room temperature at 20–25°C [3, 5, 6, 10-12, 21, 26]. Although the organism is sublethally injured during processing, the risk of campylobacteriosis may still remain, because as few as 500 cells cause illness and there is still the potential of viable but not culturable cells (VBNCs) [20]. The study considers temperature as a parameter of survival, along with the detection of *C. jejuni* in the presence of the antimicrobial agent, acetic acid.

Acetic acid is a Generally Recognized as Safe (GRAS) compound, and is believed to be a reliable agent to control *C. jejuni* [17]. The acid is safe, not costly, easily available, and also effective. Several studies have previously reported on the bacteriocidal activity of organic acids with *C. jejuni* [1, 16, 17], however, little information is currently available on the antimicrobial activity of acetic acid on chicken at different temperatures.

Accordingly, 1% acetic acid was applied as the upper limit for a kinetic inactivation study of *C. jejuni*. The detection of *C. jejuni* in a model system using chicken and acetic acid was performed by the plate count method using campylobacter selective media, and a polymerase chain reaction based on 16S rRNA primers [13] after exposed to D values at 4, 25, and 42°C. The results are compared to the results obtained from the enrichment culture and polymerase chain reaction method.

MATERIALS AND METHODS

Strains and Culture Conditions

The *Campylobacter jejuni* ATCC 33291 was maintained as a freeze-dried ampoule at 4°C. The strain was cultured microaerobically in a FBP-supplemented Brucella broth

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(FBP-SBB) [8, 21] at 42°C for 48 h in a 3.5-l anaerobic jar with a campylobacter microaerophilic system (Difco, Detroit, Michigan, U.S.A.). The FBP-SBB consisted of 0.9 mM ferrous sulfate, 1.3 mM sodium metabisulfite, and 2.3 mM sodium pyruvate in a Brucella broth (Difco). Filtrate antibiotics, vancomycin 15 mg, trimethoprim lactate 5 mg, polymyxin B 20,000 IU, cycloheximide 50 mg/l, and 3% bovine calf serum (Hyclone, Logan, Utah, U.S.A.) were separately added to the FBP-SBB after autoclaving. Either FBP-SBM (FBP-SBB with a 2% agar plus 5% defibrinated sheep blood (Komed Co. Bundang, Korea)) or a campylobacter selective agar (Lab. M Co., Bury, England) were used for the solid culture. *Escherichia coli* ATCC 25922 and *Clostridium perfringens* ATCC 13124 were cultured in a nutrient broth and differential Reinforced Clostridial media (Difco) under aerobic and anaerobic conditions, respectively [15].

D Values

FBP-SBB with 1% acetic acid was inoculated to yield a final concentration of approximately 10^7 CFU/ml of *C. jejuni*, and the suspension was incubated at 4, 25, and 42°C. After 30 s, 5 min, 30 min, and 60 min, the number of cells was determined by the plate count method. The results were plotted as a semilog graph and the D value was obtained from No (initial CFU/ml) to 0.1 No from the linear part of the death curves.

Studies Using Chicken Model System

Chicken purchased from a local supermarket was minced after removing bones. A 10 g sample containing skin and other meat parts was inoculated with 10^8 CFU/g of *C. jejuni*. The inoculated chicken was soaked in 90 ml of 1% acetic acid solution (89 ml of FBP-SBB and 1 ml of acetic acid, 167 mM acetic acid) in a 250-ml Erlenmeyer flask. The chicken in the 1% acetic acid solution was incubated at 4, 25, and 42°C in a water bath and the reduction in cells was enumerated at each determined time using 1 D to 15 D values.

Enrichment Culture

After acetic acid exposure, 3 ml of the aqueous portion from the chicken slurry was filtered using a sterile membrane filter (pore size 0.45 µm, Millipore, MA, U.S.A.). The collected cells on the filter were soaked in 30 ml of FBP-SBB with 3% serum in a 100-ml Erlenmeyer flask with a screw cap. The sample was incubated microaerobically at 42°C for 24 h and one loopful of enrichment culture solution was streaked on a campylobacter selective agar plate and incubated for 2 days. The result was estimated as positive or negative based on the colonies that appeared on the selective agar.

Preparation of Sample for PCR Method

A 1 ml sample was centrifuged at 8,000 ×g for 3 min, washed twice with sterile distilled water (SDW), and then resuspended in 10–100 µl of SDW. The washed cell suspension was directly used as the DNA template for the PCR amplification without further lysis treatment [23, 27].

Primers

The oligonucleotide primers were synthesized by a commercial company (Genotech, Taejon, Korea). The sequences of the oligonucleotide primer based on a 16S rRNA gene [13, 14] were pA: GGAGGATGACACTTTTCGGAGC and pB: ATTACTGAGATGACTAGCACCCC.

PCR Assay

The PCR was performed using 1 µl of the cell suspension in a 20 µl volume of the PCR PreMix™-*TOP* (Bioneer, Chongwon, Korea). The PCR mixture consisted of 1 U of Taq DNA polymerase, 250 µM each dNTP, 10 mM Tris-HCl (pH 8.3), 40 mM KCl, and 1.5 mM $MgCl_2$. The mixture was covered with 20 µl of sterile mineral oil in each tube and the PCR was carried out in a BioRad Gene Cycler (Model No. 10167, Japan). The PCR cycle program of denaturation, annealing, and extension temperatures was comprised of one cycle of 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, and one cycle of 5 min at 72°C. The PCR products (10 µl each) were analyzed using a 1% agarose gel in a TAE buffer containing 0.5 µg/ml of ethidium bromide. The gel was visualized and photographed under UV light.

RESULTS AND DISCUSSION

Effect of Temperature on Inhibition of *C. jejuni* ATCC 33291 by Acetic Acid

Figure 1 shows the responses of *C. jejuni* ATCC 33291 to 1% acetic acid at pHs 5.5 and 6.5 and at 4, 25, and 42°C for up to 240 h (10 days). As shown in the figure, the initial 10^7 CFU/ml level of *C. jejuni* cells decreased to a 10^3 CFU/ml within 4 h at 42°C, however, almost the same number as the initial level of 10^7 CFU/ml was maintained at 4°C and 25°C for 8 h, even with the addition of 1% acetic acid at pH 5.5 or pH 6.5. The data clearly show that the suppression of *C. jejuni* in the presence of 1% acetic acid was different depending on the temperature. In the control samples without acid, the initial number of cells, 10^7 CFU/ml, did not significantly change much at 42°C after the first proliferation for 240 h, whereas the number decreased to 10^{4-5} CFU/ml at 4°C, and decreased to below 10 CFU/ml at 25°C after 240 h. Upon the addition of 1% acetic acid, the growth of *C. jejuni* was inhibited gradually at 4 and 25°C, yet sharply at 42°C. In all samples tested, the inhibition of *C. jejuni* by acetic acid was more pronounced at pH 5.5 than at pH 6.5.

The optimal temperature for the growth of strain *C. jejuni* is 42°C and it is well known that the growth of this

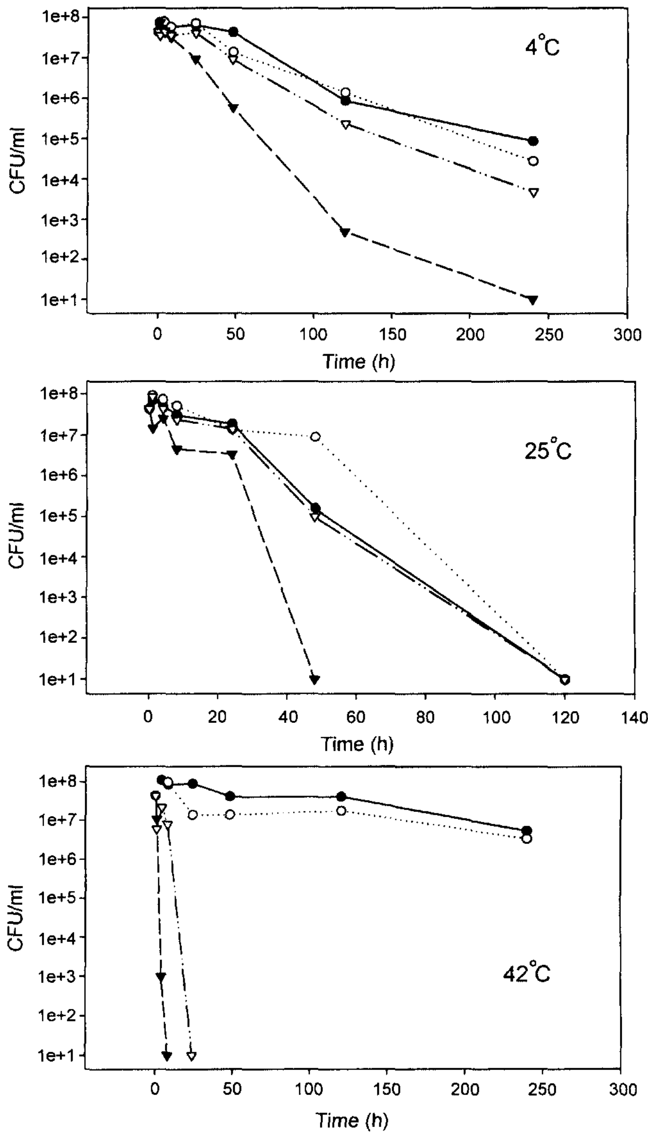

Fig. 1. Growth and suppression of *C. jejuni* ATCC 33291 in FBP-supplemented Brucella broth with 1% acetic acid during 10 days. -●- Control, pH 5.5; -○- Control, pH 6.5; -▼- Acetic acid 1%, pH 5.5; -▽- Acetic acid 1%, pH 6.5.

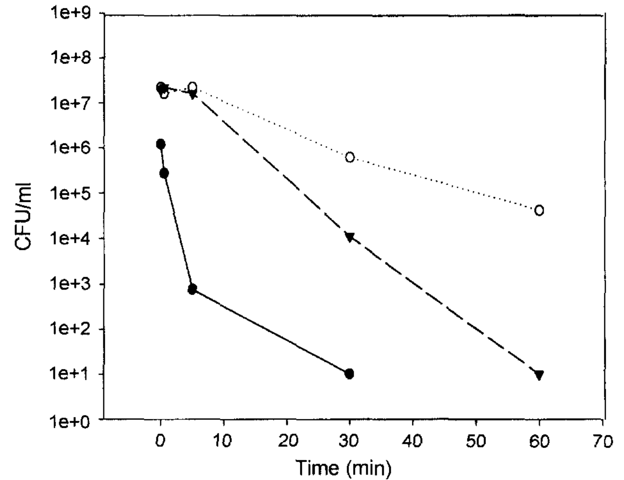

Fig. 2. Estimated death rate for *C. jejuni* ATCC 33291 with 1% acetic acid at 4, 25, and 42°C during 60 min. -○- 4°C; -▼- 25°C; -●- 42°C.

organism is reduced more at room temperature than at refrigeration temperatures, as mentioned earlier [3, 5, 6, 10-12, 21]. The resistance of the bacteria to an organic acid at a low temperature was demonstrated once again in this study with *C. jejuni*. Perales and Garcia [18] studied the influence of pH and temperature on the behavior of *Salmonella enteritidis* in homemade mayonnaise and observed a better bactericidal activity for vinegar at 35°C than 4°C. They suggested that a low temperature may afford some protection to *Salmonella* cells against the antimicrobial effects of the organic acid.

The antimicrobial activity by acetic acid on *C. jejuni* seemed to be significant for actively growing cells at 42°C. At 4°C, the organism is dormant or not actively growing, whereas the organism at 25°C may be lysed in a death condition for certain reasons. It has been suggested that the antibacterial activity of organic acids acts as an uncoupler for bacteria [19]. As such, they dissipate the electrochemical gradient of the cell membrane as well as denature the protein inside the cells at a lower pH. In this sense, at 42°C, acetic acid may interfere with the nutrient transport and energy generation, thereby resulting in the growth inhibition of the energized *C. jejuni* cells. However, such an antimicrobial action at 42°C would not appear to function in the suppressed phase of *C. jejuni* cells at 4°C. These results indicate that the use of acetic acid to control *C. jejuni* should be selectively applied, depending on the temperature of food sanitation. Also, the concentration of acetic acid and period of time should be carefully applied, especially at 4°C, a common refrigeration temperature.

The death rate curves for *C. jejuni* ATCC 33291 in the presence of 1% acetic acid at 4, 25, and 42°C without adjusting the pH are shown in Fig. 2. This experiment was designed to confirm the tolerance of *C. jejuni* at 4°C under 1% acetic acid at pHs 5.5 and 6.5, as shown in Fig. 1. A 10^7 CFU/ml level of *C. jejuni* cells was inoculated into FBP-SBB media including 1% acetic acid, and the viabilities were compared at 4, 25, and 42°C after 60 min. The cells died rapidly at 42°C and viable cells were no longer detected after 30 min, using the plate counting. At 4 and 25°C, the death rates were considerably slow. At 4°C, the organism survived for 60 min at 10^4 CFU/ml. The sensitivity of *C. jejuni* to 1% acetic aicd in this test was in agreement with the results at pH 5.5 and pH 6.5, showing the order of 42>25>4°C. From the data of Fig. 2, the D values of *C. jejuni* in the FBP-SBB media with 1% acetic acid were estimated as 22, 8.5, and 1.4 min at 4, 25, and 42°C, respectively.

Several previous studies have suggested that the addition of organic acids, such as acetic acid or lactic acid, is very effective to reduce *C. jejuni* contamination [1, 15-17, 22]. Netten *et al.* [16] reported that decontamination with 1% lactic acid at pH 3.0 and 21°C for at least 30 s is effective for *C. jejuni.* The D values obtained from other similar studies are shorter than the values estimated in the current study [16, 17]. The reason for this difference was that the all experimental conditions, including the acids and media as well as the strain used, were different. After the addition of 1% acetic acid, the pH of the media was 3.94. The organoleptic characteristics of the meat, such as the odor and color, could deteriorate at this pH. One % acetic acid was used for further experiments with a system using chicken.

Effect of Medium on Growth Inhibition of *C. jejuni* ATCC 33291 in Model System Using Acetic Acid and Chicken

Data on the survival and killing of *C. jejuni* in a system containing FBP-SBB media or distilled water at 4, 25, and 4°C are presented in Tables 1 and 2. The D values used were from Fig. 2. In this experiment, an enrichment culture and polymerase chain reaction (PCR) were used to obtain data to supplement that obtained from plate count.

At 42°C in a model system with FBP-SBB media (Table 1), the cell death from zero time to 3 D_{42} (number before D means times of D values at each temperature) was insufficient to be detected, and then cell death occurred from 3 D_{42} to 5 D_{42} on a decimal level. When the number of cells reached 10^2 CFU/ml after exposure to 7 D, there was no further cell death until 15 D_{42}. Overall, in the model system with chicken, the rate of death of *C. jejuni* was quite low, compared with the media conditions containing only acetic acid. This phenomenon was the same at 4°C and 25°C (Table 1). The organisms were viable from 10^1 to 10^7 CFU/ml, as enumerated by the plate count method. The amplification of the campylobacter-specific 426 bp 16S rRNA gene was detected in the tested samples by PCR, as expressed in the second section in Table 1.

By using pA and pB primers and washed whole cells of *C. jejuni* ATCC 33291, the detection limit by PCR was estimated to be the level of 10 cells [21]. Such numbers of *C. jejuni* in all samples were sufficient to be detected by the PCR, as shown in Table 1.

Usually, the death rate of bacteria becomes slow with the addition of food components [19]. Okrend *et al.* [17] reported that the addition of FBP as supplements for *C. jejuni* increased the D_{52} value from 5.97 to 10.93 min, showing protection by FBP. Accordingly, it would appear that the chicken components also strongly influenced the reduction in the death rate of the bacteria. Thus, the combination of the components in the FBP-SBB media and the chicken in the model system seemed to support the survival of *C. jejuni* in the presence of acetic acid. To confirm the results, distilled water was substituted for the FBP-SBB media. As shown in Table 2, at 42°C, the organisms inoculated at 10^7 CFU/ml were not detected by the plate count method even at zero time, which consumed approximately 10 s. The organism was detected by PCR from a sample which had 10^{-9} CFU/ml, theoretically calculated after the exposure to a 15 D_{42} value for 21 min

Table 1. Survival of *C. jejuni* ATCC 33291 under D value exposed in model system with FBP-SBB medium broth

D-value*		Ctr.**	0	1 D	3 D	5 D	7 D	9 D	11 D	13 D	15 D
Practical	Theoretical number of cells expected	10^7	10^6	10^5	10^3	10^1	10^{-1}	10^{-3}	10^{-5}	10^{-7}	10^{-9}
Detection method	Temp. (°C)										
Plate count (CFU/ml)	4	1.0×10^7	6.0×10^5	6.0×10^4	2.2×10^3	1.6×10^3	8.8×10^2	4.6×10^2	2.4×10^2	1.4×10^2	2.0×10^1
	25	5.0×10^6	2.0×10^4	2.4×10^4	7.0×10^3	7.0×10^2	4.0×10^3	2.0×10^2	1.2×10^2	1.6×10^2	2.0×10^1
	42	3.4×10^7	7.2×10^6	2.0×10^6	1.8×10^5	1.6×10^3	4.0×10^2	2.4×10^2	6.0×10^2	1.2×10^2	1.8×10^2
PCR***	4	+[a]	+	+	+	+	+	+	+	+	+
	25	+	+	+	+	+	+	+	+	+	+
	42	+	+	+	+	+	+	+	+	+	+
Enrichment culture	4	/[b]	/	/	/	/	/	+	+	+	+
	25	/	/	/	/	/	+	+	+	+	+
	42	/	/	/	/	/	+[b]	+	+	+	+

*D values exposed at D_4=22, D_{25}=8.5, and D_{42}=1.4 min for the model system with 1% acetic acid and 10% chicken in FBP-SBB.
**Ctr: Seed culture.
***PCRs were performed in 20 µl of a PCR reaction mixture using 10 pmole of each pA and pB primer based on the 16S rRNA gene, and the amplification products were confirmed at 426 bp by electrophoresis.
[a]+, 426 bp amplified product detected.
[b]/, not tested; +, growth.

Table 2. Killing of *C. jejuni* ATCC 33291 in a model system with distilled water.

D value*		Ctr.***	0	1 D	3 D	5 D	7 D	9 D	11 D	13 D	15 D
Practical	Theoretical number of cells expected	10^7	10^6	10^5	10^3	10^1	10^{-1}	10^{-3}	10^{-5}	10^{-7}	10^{-9}
Detection method	Temp. (°C)										
Plate count (CFU/ml)	4	3.6×10^6	2.8×10^6	<10	<10	<10	<10	<10	<10	<10	<10
	25	4.6×10^6	<10	<10	<10	<10	<10	<10	<10	<10	<10
	42	1.8×10^7	<10[a]	<10	<10	<10	<10	<10	<10	<10	<10
PCR	4	+	+	+	+	+	+	+	+	+	+
	25	+	+	+	+	+	–[b]	Δ[b]	+	–	–
	42	+[b]	+	+	+	+	+	+	+	+	+
Enrichment culture	4	/	+[C]	/	/	/	–	+	+	–[C]	–
	25	/	/	/	/	/	+	–	–	–	–
	42	/[C]	/	/	+	/	/	+	/	/	–

*D values exposed at D_4=22, D_{25}=8.5, D_{42}=1.4 min for the model system with 1% acid and 10% chicken in distilled water.
**PCRs were performed in 20 µl of a PCR reaction mixture using 10 pmole of each pA and pB primer based on the 16S rRNA gene, and the amplification products were confirmed at 426 bp by electrophoresis.
***Ctr: Seed culture.
[a]<10, no colony detected on selective media inoculated with 0.1 ml of broth
[b]+, 426 bp amplified product detected; –, not detected; △, no clear band.
[c]/, not tested; +, growth; -, no growth.

(second section in Table 2). The pH of the model system with distilled water was 3.31. However, the rapid death of *C. jejuni* in distilled water was not solely dependant on the lower pH, as it was found that components in the environment also had a strong effect on the survival of the organism in the model system at the same pH conditions. Regardless of the viability, the DNA of *C. jejuni* was normally amplified, even after the organism was damaged by 1% acetic acid at a low pH in a distilled water environment. The organisms not detected by the plate count method were detected in certain samples through an enrichment culture (third section in Table 2), thus indicating that the injured cells were repaired in enrichment media.

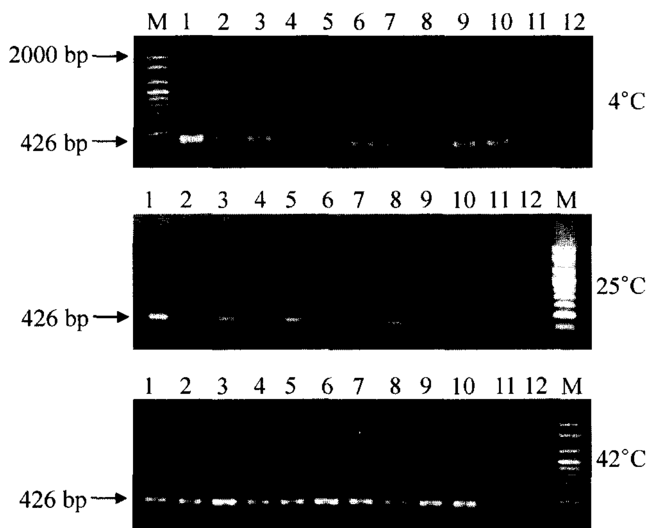

Fig. 3. Detection of *C. jejuni* ATCC 33291 by PCR exposed to D values in a model system.
The PCR was performed with 10 pmole of pA and pB primers using washed whole cells from the model system with 1% acetic acid and 10% chicken in distilled water at 4, 25, and 42°C. The initial number of cells before exposure to the D values was at a level of 10^5 per PCR. Lane 1, control; 10^5 cells of *C. jejuni*; lane 2, zero time; lane 3, 1 D; lane 4, 3 D; lane 5, 5 D; lane 6, 7 D; lane 7, 9 D; lane 8, 11 D; lane 9, 13 D; lane 10, 15 D; lane 11, blank (no DNA); lane 12, reference: *E. coli* (4°C and 42°C) and *Cl. perfringens* (25°C); M, DNA size marker.

Anderson [1] and Netten *et al.* [16] have suggested that the antibacterial effect of acid decontamination depends primarily on immediate death. However, there is no evidence on whether immediate death has a potential pathogenesis which can be repaired by an enrichment process. Therefore, to properly use acetic acid, further study is necessary to understand the repairable activity of the cells suppressed by antimicrobial substances in food sanitation.

In conclusion, the rates of death of *C. jejuni* in the model system with chicken did not follow linear relationships observed with media of low pH conditions. This is an important point when considering the use of acetic acid. In addition, since the detection of bacteria by PCR does not necessarily indicate the organism's viability, the PCR method provides only the potential of contamination rather than the exact survival or lethality of the bacteria. The PCR technique is sensitive and specific in detecting pathogens in food, however, the above-mentioned characteristics of the PCR method can be a limitation. Alternatively, the PCR method can be applied as an expandable detection technique in the field of food sanitation.

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