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ORIGINAL ARTICLE

Effect of low concentrations of lactic acid and temperature on the expression of adhesion, invasion, and toxin-encoding genes of *Campylobacter jejuni* from poultry

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ABSTRACT

Background and Objectives: The consumption of contaminated poultry meat is considered as a significant route of campylobacteriosis transmission. Lactic acid is a disinfectant agent with bactericidal effects on *Campylobacter* spp. The purpose of this study was to assess the low concentrations of lactic acid effect and different temperatures on the transcriptomic responses of *Campylobacter jejuni (C. jejuni)* adhesion and virulence-associated genes including *peb4*, *ciaB*, *cdtA*, *cdtB*, and *cdtC*.
Materials and Methods: The samples were incubated at 10°C and 22°C for 48 h upon exposure to 30% and 60% lactic acid. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of lactic acid was also determined. Then, gene expression was assessed using real-time polymerase chain reaction (RT-PCR).
Results: Lactic acid had lower MIC and MBC levels at lower temperature. The utilization of both levels of lactic acid significantly reduced the expression of *peb4*, *ciaB*, *cdtB*, and *cdtC* genes over 48 h of incubation at 22°C. However, no significant difference was found in the expression of the *cdtA* gene between 10 and 22°C at 30% lactic acid.
Conclusion: These results highlight the potential of low-concentration lactic acid in the downregulation of adhesion and virulence-associated genes as well as reduction of *C. jejuni* pathogenicity.

Keywords: *Campylobacter jejuni*; Colonization; Gene expression; Virulence factors

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INTRODUCTION

Poultry meat is considered as the main source of daily animal protein intake due to relatively low cost of production, high efficiency, lack of serious common zoonotic diseases, nutritional superiority of white meat over red meat, and short breeding period compared to other livestock (1). However, the poultry meat and its products are considered as the most nutritive matrices for supporting *Campylobacter* spp. growth (2-4). Due to increasing human foodborne infections, mainly gastroenteritis caused by *Campylobacter* species, and resistance to antimicrobial agents, control of the quality of products throughout the processing, production, and distribution chains are of great importance to prevent bacterial foodborne diseases (3, 5-7). *C. jejuni* is a Gram-negative, spiral-shaped microaerophilic bacterium reported as the most common foodborne pathogen throughout the world. High temperature (37-42°C) and low oxygen conditions (approximately 5%) are the optimum environmental factors to support the bacterial growth (5). Post-process contaminations and the consumption of semi-cooked and/or raw poultry meat are the major risk factors for human campylobacteriosis (5). Several studies have reported the prevalence of *C. jejuni* in various food matrices such as chicken, turkey, and duck meat (8), beef, lamb, as well as their products (9, 10), along with mutton and buffalo meat (11), fish, cattle, and sheep meat (1), as well as pork (10, 12). Various substances such as natural alternatives (plant-derived extracts (13-15), synthetic chemicals, probiotics (16) and organic acids (15, 17) have been applied in addition to antibacterial agents to reduce the burden of human *Campylobacter* infection (15). Their main mechanisms of action include inhibition of bacterial attachment to intestinal epithelial cells and invasion and down-regulation of virulence-associated factors such as enterotoxins (5). Briefly, lactic acid is one of the large-scale chemicals produced via fermentation by probiotic bacteria, molds, and yeasts. In an un-dissociated form, lactic acid attacks and easily penetrates the bacterial cell membrane. In such circumstances (in the dissolved state), it is dissociated and may inhibit *C. jejuni* growth through different mechanisms involving enhancement of cell permeability, creation of channels, ions exchange, H+ depletion, changes in cellular ATP, nucleic acids, and proteins. Additionally, lactic acid is metabolized in the cytoplasm and disrupts the activity of cellular enzymes such as carboxylase and catalase as well as the food transport system (18, 19). Multifactorial processes involving motility, attachment, colonization, invasion, and toxin production are involved in the pathogenesis of *C. jejuni* (20). These processes are strongly influenced by various intrinsic and extrinsic parameters. Bacterial attachment (CadF), cell invasion (CiaB), and toxin production (CdtA, CdtB and CdtC and Peb4) are considerable (21-23). Among all mentioned substances, using weak organic acids are popular and cost-effective (24). It is well known that organic acids are generally recognized as safe substances. Lactic acid as a natural and non-toxic antimicrobial molecule is extensively used in the food industry for the decontamination (17). It has a hydrophilic structure and low molecular weight (nearly 90 Da), facilitating its penetration into the microorganism's membrane. Therefore, the cell membrane's permeability is increased as a result of intracellular acidification and integrity modification (25). However, its utilization is restricted due to off-flavor and other undesirable attributes. Hence, it is suggested that the lower concentration of lactic acid in combination with other materials and/or technologies could control the pathogenicity and achieve to better overall acceptance (26). For instance, the cold storage of chicken pieces treated with lactic acid not only reduced the population of *C. jejuni* but also prolonged specimens' shelf life (27).

Although, there are numerous studies dealing with the incidence of *Campylobacter* species in poultry meat, little information is available in this regard (1, 5, 11, 12). There is not sufficient literature review regarding the combined effect of lactic acid and temperature against *C. jejuni* in chicken meat. Thus, this study aimed to evaluate the influence of lactic acid and temperature on the expression of adhesion and virulence genes of *C. jejuni* inoculated onto chicken broth.

MATERIALS AND METHODS

Bacterial strain preparation. The *C. jejuni* strain ATCC 29428 was provided from Razi Vaccine and Serum Research Institute (Karaj, Iran) as an active culture. The stock of *C. jejuni* strain under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N) at -70°C in tryptic soy broth (TSB) (Merck, Germany) containing 30% glycerol was used to revive the isolates in brain heart infusion (BHI) broth (10 mL,

Merck, Germany) at 37°C for 24 h. Next, the bacterial pellets were separated through centrifugation at 5000 rpm for 10 min, followed by re-suspending in a sterile physiological serum. The optical density (OD) of 0.08-0.1 (at 600 nm) or 10^8 CFU/mL *C. jejuni* was confirmed by plate count (28).

Preparation of chicken broth. Fresh chicken meat carcasses were washed and transported immediately from the slaughterhouse to the laboratory under cold conditions. The chicken meat broth was prepared according to Pilevar study (28), with minor modifications. Briefly, the chicken meat was minced twice and mixed with sterile distilled water in equal proportions (w/w). After mixing, the supernatant was collected through centrifugation at 5000 rpm for 10 min. The resultant liquid was autoclaved at 121°C for 15 min and stored at 4°C until testing.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Lactic acid (pH 2.43; Merck, Germany) was purchased from a local market. The MIC of lactic acid in chicken broth was determined using the broth macro-dilution method (BHI medium) at different temperatures (10, 22, and 42°C) (Table 1) (29). For serial dilutions (nine tubes), minced chicken meat samples (5 g) were blended with 45 mL of physiological serum. Then, 1 mL of each 30% and 60% dilutions was pour plated in *Campylobacter* Selective Agar (CAMPY) (Merck, Germany) and incubated at 37°C for 48 h. Finally, the count number of *C. jejuni* was enumerated. For bacterial inoculation, 10^8 CFU/mL *C. jejuni* (equivalent to OD: 0.08-0.1) suspension was supplemented in falcon tubes, which contained 30% and 60% concentrations of lactic acid. The positive control confirmed the survivability of bacterial cells, while the sterility was checked by the negative control. Then, each tube was incubated at 10°C, 22°C, and 42°C for 48 h corresponding to the refrigerator, room, and optimum growth temperatures, respectively. Ultimately, the MIC to chicken broth was calculated as the lowest concentration (µg/mL) inhibiting the growth of initial inoculated bacterial cells (10^8 CFU/mL). For MBC measurement, the tubes without turbidity, meaning no visible colony growth were inoculated in PCA (Plate count agar) (Merck, Germany) and were maintained for the next 48 h at 10°C, 22°C and 42°C. The minimum concentration of lactic acid suppressing *C. jejuni* growth was considered as MBC in µg/mL.

Preparation of treatments. The inoculated chicken broth samples consisting of two levels of sub-MIC of lactic acid i.e., 30% and 60% stored at 10°C (a common refrigeration abuse temperature) and incubated at 22°C (as the ambient temperature) were designated as S1, S2, S3, and S4. These treatments refer to the inoculated chicken broth with 30% lactic acid stored at 10°C (S1), the inoculated chicken broth with 60% lactic acid kept at 10°C (S2), the inoculated chicken broth with 30% lactic acid incubated at 22°C (S3), and inoculated chicken broth with 60% lactic acid maintained at 22°C (S4) (30).

RNA extraction. Bacterial RNA extraction was performed using the RNA extraction kit (GeneAll Biotechnology Co., Ltd, Korea) as per the protocol. The quality and quantity of the extracted RNA was

Table 1. Primer sequences used in this study

Gene	Primer	Sequence (5`-3`)	Amplicon Size (bp)	Reference
16SrRNA	Forward	TGCTAGAAGTGGATTAGTGG	153	
	Reverse	GTATTAGCAGTCGTTTCCAA		
Peb4	Forward	AAGGTGGTGAGCTTGGTTGG	123	
	Reverse	TTAAGCGCGAAAGCAGCATC		
CiaB	Forward	AAAAGCTTGGCAAGAAGCTG	107	(5)
	Reverse	ATGCCACCGCATGAGTATAA		
CdtA	Forward	GGATTTGGCGATGCTAGAGTT	147	
	Reverse	CATTTGTGCGTGATTGCTTG		
CdtB	Forward	CTGGATAGCAGGGGATT	110	
	Reverse	CTTGAGTTGCGCTAGTTGGA		
CdtC	Forward	TCAGCTGTGCAAATTCGTTC	121	
	Reverse	AAATAGGATCTAGGGTGCAAGG		

determined using the NanoDrop® spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 260 nm and 280 nm, respectively. All impurities in the obtained RNA such as protein and other contaminations were identified by A260/280 and A260/230 parameters.

cDNA synthesis. The cDNA was synthetized from RNA by reverse transcription, according to the PrimeScript™ RT reagent kit (Takara Shuzo, Kyoto, Japan), following manufacturer's instructions, and was stored at refrigerator temperature until needed. For each 10 µL reaction mixture, 2 µL of primer 5×PrimeScript™ buffer solution, 0.5 µL of PrimeScript™ RT Enzyme Mix I, 0.5 µL of Oligo dT primer (50 µM), 0.5(100 µM), and 6.5 µL of total RNA and RNase-free dH_2O were used. The reaction mixture was prepared on ice and then incubated at 37°C for 15 min for reverse transcription and at 85°C for 5 s to inactivate the enzyme with heat treatment.

Real-time PCR. The expression of adherence, virulence, and cytotoxin-producing genes (*peb4, ciaB, cdtA, cdtB,* and *cdtC*) of *C. jejuni* was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR) technique. The gene 16S rRNA was used as the internal control. The reaction components included 20 µL of tube volume containing 10 µL of SYBR Premix Ex TaqII (2x) (Takara Bio Inc., Otsu, Japan), 0.4 µL of each primer (Table 2), 0.4 µL Rox reference dye, 2 µL reaction solution (cDNA solution), and 6.8 µL of sterile distilled water. The thermal cycling conditions for qRT-PCR (25 µL reaction volumes) included an initial denaturation of 15 min at 95°C, 25 s at 95°C, 30 s at 60°C, and 30 s at 72°C (21). The primer specificity was determined by melting curve analysis. The qRT-PCR assays were performed in quadruplicate (Table 1). The calculation of relative gene expression was performed using $2^{-\Delta\Delta Ct}$ method. The experiment was performed in triplicate.

Table 2. MIC and MBC of lactic acid in chicken broth medium

Temperature (°C)	MIC (µg/mL)	MBC (µg/mL)
42	2.5	5
22	1.25	2.5
10	1.25	2.5

Statistical analysis. All data was analyzed by SPSS (version 25.0, SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to check the normal distribution of variables (lactic acid concentrations, time and temperatures) prior to analysis. To evaluate the influence of lactic acid, incubation time, and temperature, ANOVA test with Duncan's post hoc test was employed at the confidence level of 95%. Mean ± standard deviation expresses the values.

RESULTS

MIC and MBC. The MIC and MBC results have been represented in Table 2. Accordingly, lower temperatures decreased the MIC and MBC levels of lactic acid.

Adhesion gene expression in chicken broth samples exposed to acidic stress. As shown in Fig. 1, lactic acid supplementation significantly affected expression of the *peb4* gene related to adherence property of *C. jejuni* in treated samples ($p<0.05$). The changes in the expression of *peb4* gene during the incubation time followed an ascending trend; the minimum *peb4* gene expression was observed in S1 and S4 specimens after 24 h of storage, while gene expression variation in S3 sample was maximum at the same time (Fig. 1,

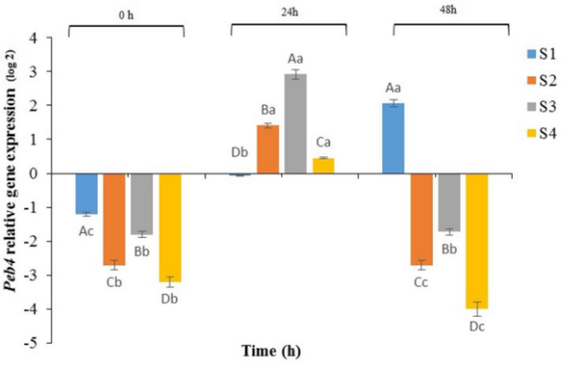

Fig. 1. Effect of lactic acid on the expression of *peb4* gene in chicken broth medium incubated at two different temperatures for 48 h. A-D different letters within the supplemented treatments in the same time units (A is the highest and D is the lowest gene expression) and a-d different letters within the comparisons between different incubation times (0, 24 and 48 h) (a is the highest and d is the lowest gene expression) indicate statistically significant differences at $p<0.05$. Treatments: S1: chicken broth+30% lactic acid stored at 10°C, S2: chicken broth+60% lactic acid stored at 10°C, S3: chicken broth+30% lactic acid stored at 22°C, S4: chicken broth+60% lactic acid stored at 22°C.

24 h). In addition, the expression of the *peb4* gene was reduced except for S1 (24-48 h) ($p<0.05$).

Changes in expression of invasion gene in chicken broth samples exposed to acidic stress. As depicted in (Fig. 2, the impact of lactic acid concentration, incubation time, and temperature on the expression of the *ciaB* gene of treated isolates during storage was observed. A reduced *ciaB* gene expression was displayed in S1 and S2 samples ($p<0.05$). Interestingly, two specimens of S3 and S4 showed a rise and fall through 48 h storage. On the second day of incubation, the lowest mean values of gene expression were found in S2 and S3 samples, while the intermediate and highest level were observed for S4 and S1, respectively (Fig. 2). Notably, there was no remarkable differences in virulence gene expression between S1 and S2 specimens at the initial time of incubation.

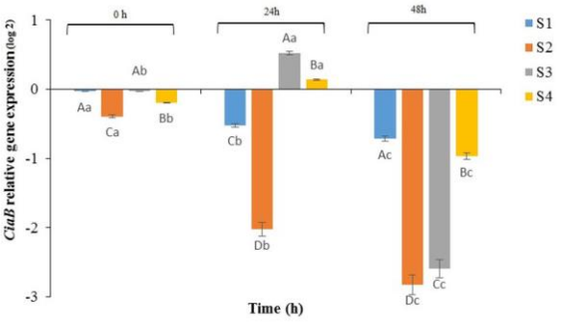

Fig. 2. Effect of lactic acid on the expression of *ciaB* gene in chicken broth medium incubated at two different temperatures for 48 h. A-D different letters within the supplemented treatments in the same time units (A is the highest and D is the lowest gene expression) and a-d different letters within the comparisons between different incubation times (0, 24 and 48 h) (a is the highest and d is the lowest gene expression) indicate statistically significant differences at $p<0.05$. Treatments: S1: chicken broth+30% lactic acid stored at 10°C, S2: chicken broth+60% lactic acid stored at 10°C, S3: chicken broth+30% lactic acid stored at 22°C, S4: chicken broth+60% lactic acid stored at 22°C.

Effect of acidic stress on toxin production by *C. jejuni*. The influence of triple factors of acidic condition, incubation time, and temperature on transcription levels of virulence-associated genes in *C. jejuni* was exhibited in Fig. 3. The incorporation of chicken broth with desirable levels of lactic acid incubated at two different temperatures over time indicated that the toxin production of treated specimens was mark-

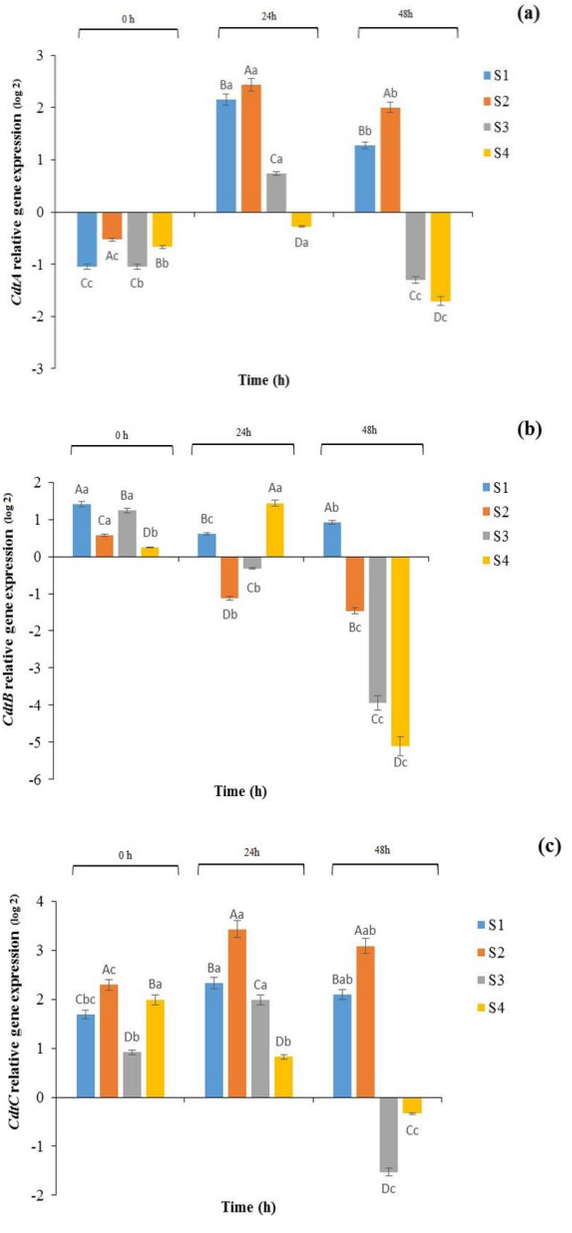

Fig. 3. Effect of lactic acid on the expression of *cdtA* (a), *cdtB* (b), and *cdtC* (c) genes in chicken broth medium incubated at two different temperatures for 48 h. A-D different letters within the supplemented treatments in the same time units (A is the highest and D is the lowest gene expression) and a-d different letters within the comparisons between different incubation times (0, 24 and 48 h) (a is the highest and d is the lowest gene expression) indicate statistically significant differences at $p<0.05$. Treatments: S1: chicken broth+30% lactic acid stored at 10°C, S2: chicken broth+60% lactic acid stored at 10°C, S3: chicken broth+30% lactic acid stored at 22°C, S4: chicken broth+60% lactic acid stored at 22°C.

edly affected by the concentration of lactic acid, time, and temperature ($p<0.05$) (Fig. 3).

Concerning the role of lactic acid concentration, incubation time, and temperature on the *cdtA* gene expression, a mixed regulatory reply was seen over time (Fig. 3a). The maximum *cdtA* expression after 24 h incubation was observed in the S2, S1, and S3 specimens compared to S4 sample, showing a descending trend until the end of storage period ($p<0.05$). The changes in the S3 and S4 samples were more substantial than in other samples, which experienced the lowest level of gene expression on the ultimate day of storage ($p<0.05$, 48 h). Further, no significant difference was found in the expression of *cdtA* gene between S1 and S3 at the initial time of storage.

It was estimated that the level of the *cdtB* gene expression in two samples of S2 and S3 continuously declined over time ($p<0.05$) (Fig. 3b). Considering S4, the expression of *cdtB* gene associated with cytotoxin efficiency considerably ($p<0.05$) enhanced on the first day of storage. However, from this point onwards, it revealed a downward trend (24-48 h) ($p<0.05$). Note that, in S4 treatment, upon elevating temperature, the antimicrobial efficiency of lactic acid dramatically increased, which was well reflected in the *cdtB* gene expression after 48 h of incubation. In the case of S1, a relative decrement and increment trends (0-48 h) can be viewed over time ($p<0.05$).

As illustrated in Fig. 3c, the *cdtC* gene expression was progressively elevated in S1, S2 and, S3 samples up to 24 h, followed by a slight reduction merely in S1 and S2 ($p<0.05$). In this regard, no significant changes were recognized in S1 treatment between 24 and 48 h as well as 0 and 48 h ($p>0.05$). Also, the differences in the amount of the *cdtC* gene expression in S2 sample (24-48 h) was insignificant ($p>0.05$). Surprisingly, the reduction in S4 over 48 h ($p<0.05$) was observed. The mean values of -1.52 and -0.33 in the *cdtC* gene expression were the lowest in S3 and S4, respectively in comparison with other samples throughout the storage period (0-48 h) ($p<0.05$).

DISCUSSION

In this study, the MIC and MBC levels of lactic acid was lower at lower temperatures. S4 conditions (60% lactic acid and 22ºC) decreased the peb4 gene expression more significantly. However, *ciaB* was higher affected by 60% lactic acid at 10ºC (S2). In addition, *cdtA*, *cdtB* and *cdtC* genes expression was considerably decreased in 60% lactic acid and 22ºC (S4), 30% and 60% lactic acid at 22ºC (S3 and S4) and 30% lactic acid at 22ºC, respectively. Microorganisms can react to environmental alterations or stressful conditions via regulating gene expression and producing various metabolites which interfere in their resistance, virulence and adaptation (31). However, *C. jejuni* turn into less virulent agent or alter gene expression in exposure to modified growth conditions (32, 33). Peb4 is an important adhesin for bacterial attachment and biofilm formation (23). The proper conditions for the virulence genes expression inhibition by lactic acid remains to be uncovered. Wang et al. suggested that, *C. jejuni* could be strongly prevented by lactic acid bacteria (LAB) due to their antagonistic activity. Indeed, *L. plantarum* and *L. casei* as a main producer of lactic acid make *C. jejuni* more susceptible to higher acidic conditions. Thus, their bactericidal capacity would be realized through reducing the capability of *C. jejuni* to adhere and invade to gastrointestinal cells as a goal texture (16). As reported by Blevins et al. lactic acid could be incorporated with water in the washing, rinsing, and/or chiller tank systems, where the poultry carcasses immersed for a duration of 45-110 min to decrease their microbial load (34). Bai et al. demonstrated the lowest concentration of lactic acid for inactivating *C. jejuni* in chicken slaughterhouse particularly in pre-cooling procedure and segmentation section was 0.025%. It has been also stated that the application of approximately 5% lactic acid is necessary for the maximum bactericidal efficiency (25). Gonzalez-Fandos revealed that the number of *C. jejuni* colonies are lower in chicken legs immersed in 2% lactic acid and then packed in modified atmospheres (40% CO_2, 60% N2) than in those washed with the same level of lactic acid and packed in fresh air. Moreover, the combined effect of modified atmosphere packaging and lactic acid could result in extended refrigerated shelf life of chicken legs (17). On the other hand, the expression of the peb4 gene is a time and temperature dependent parameter (35, 36).

The *ciaB* gene is probably a temperature regulated gene; it was downregulated at temperatures below the optimum conditions. As reported by Pilevar et al. the addition of essential oils into the minced fish medium can heavily influence on the expression of virulence genes of *Listeria monocytogenes* (28). Another study proposed that the virulence gene expression of

L. monocytogenes is chiefly affected by two factors of time and exposure to different temperatures (37). Also, they recorded higher virulence capacity of *L. monocytogenes* at 20°C in contrast with 4°C. As revealed by the current assessment, the virulence of *C. jejuni* possibly increased through addition of higher concentrations of lactic acid accompanied by higher incubation time and temperature. These findings were consistent with the results by Raeisi study that found the higher expression of *ciaB* gene in *C. jejuni* (1).

The pathogenicity of *C. jejuni* is highly dependent on the expression of *cdtA, cdtB,* and *cdtC* expression involved in cytotoxin production (21, 22). Indeed, the enhancement of invasive properties in two treatments of S1 and S2 on the first day of incubation in comparison with the primary hour may be emphasized on the restorability of pathogenicity of bacteria over time (38). In this regard, a vast majority of microorganisms might be protected against a severe stress by the cross-protective effect (39), whereby they employ adaptive mechanisms such as acid adaption and salt tolerance to resist the inappropriate conditions (40). Cross-protection is a phenomenon that whenever bacterial cells are exposed to non-lethal stresses; they exhibit noticeable resistance when the same or different stress is applied later. Apparently, the synergistic effect of pH reduction is induced by lactic acid over time as well as temperature may lead to suppression of bacterial growth and prevention from the formation of secondary metabolites such as cytotoxin. In parallel, Silveira et al. introduced a mixture of sophorolipid and lactic acid as a new sanitizer to enhance food safety at a MIC of 1% and 0.07% respectively. This combination inhibited *C. jejuni* growth and inhibited its activity immediately (25).

The lower level of the *cdtB* gene expression implied the toxicity decrease under inappropriate environmental conditions (31). As demonstrated by Koolman et al., the *cdtB* gene may have a different promoter than the other two *cdtA* and *cdtC* genes. The *cdtB* gene expression in other strains can be also assessed in various types of stress (5). Accordingly, various reactions to stressful conditions including up- or down-regulated responses have been detected in the different strains of *C. jejuni* (5, 41-43). In spite of other investigations claiming that *cdt* genes were co-transcribed, Carrillo and Koolman studies proposed that in some *Campylobacter* spp. strains the *cdt* genes may be freely expressed without dependency to each other (5, 44). Regarding the *cdtC* gene expression, 30% lactic acid and 22ºC conditions exerted downregulatory role. The vast variability of conditions for the decrease in virulence genes expression needs further verification. The limitations of this work mostly included lack of sufficient bacterial isolates and strains, and narrow lactic acid concentrations assessed for the gene expression.

CONCLUSION

The exposure of chicken broth to lactic acid at 22°C for 48 h significantly reduced the expression of the *peb4, ciaB, cdtA, cdtB,* and *cdtC* genes of *C. jejuni*. In addition, incubation temperature above or below the optimum condition could be an effective factor in bacterial growth and virulence genes expression. However, decreasing or increasing the concentration of lactic acid should be verified in the maintenance of food. Thus, due to the importance of pathogenicity of *C. jejuni*, which has raised lots of concern lately, the beneficial application of lactic acid can be uncovered in control of the growth of *C. jejuni* and reducing its virulence potential in chicken meat products. More comprehensive information is required to evaluate the role of environmental parameters in the expression and regulation of virulence genes of *C. jejuni*.

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