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Original Research

Isolation and Characterization of Arcobacter butzleri from Environmental Samples and Determination of their Pathogenic Gene Expression under Different Physicochemical Conditions

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Abstract

Being transmitted through food and water, *Arcobacter butzleri* (*A. butzleri*) is known as an emerging pathogenic microorganism in humans. This Campylobacter-like organism can cause infertility, miscarriage, as well as gastrointestinal (GI) disorders in animals and even lead to gastroenteritis, bacteremia, endocarditis, and peritonitis in humans. To meet the research objectives, a total number of 384 samples were collected from the wastewater discharged by a poultry slaughterhouse (n = 238) and the Cheshmeh Kileh River (146) in the city of Tonekabon, northern Iran, during the four seasons of the year. Then, the samples were evaluated using culture techniques and phenotyping. In addition, the polymerase chain reaction (PCR) was applied to confirm *A. butzleri* via 16S ribosomal ribonucleic acid (rRNA) sequencing and pathogenic genes, i.e., tlyA, ciaB, and mviN. Afterwards, the effect of various environmental stresses on the isolates was evaluated. Finally, the reverse transcription (RT)-PCR was exploited to reflect on expression/lack of expression of the pathogenic genes at pre-/post-shock stages. Totally, 58 isolates were established as *A. butzleri*, which contained three pathogenic genes out of all three strains. The effect of various environmental stresses on tlyA, mviN, and ciaB expressions also demonstrated discontinued expression at pH levels of 5.0, 6.0, and 9.0; 5% sodium chloride (salt, NaCl) concentration; and 90 s ultraviolet (UV) light exposure time. In addition, the expressions of tlyA and ciaB stopped at 20 and 40ºC while no temperature affected the expression of mviN.

Keywords: *Arcobacter butzleri*, environmental stress, virulence gene, phylogenetic analysis

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Introduction

Arcobacter butzleri (*A. butzleri*) is known as a Gram-negative, curved, spiral-shaped, non-spore-forming, and motile bacterium of the family Campylobacteraceae [1]. This microorganism causes infertility, miscarriage, as well as gastrointestinal (GI) disorders in animals and even leads to gastroenteritis, bacteremia, endocarditis, and peritonitis in humans [2]. In addition, *A. butzleri* may induce septicemia, abdominal cramp-like pains, and chronic diarrhea for more than two months [3, 4]. *A. butzleri*, as the most common species, is widely found in water, which is the source of animal food. In addition, it is usually isolated from a variety of environments such as beef, pork, poultry, lettuce, water and drainage tanks, milk and animal feces [5, 6].

In this regard, obvious resistance of *A. butzleri* along with its ability to survive and grow in aerobic conditions augments its potential as a food- and water-borne pathogen. On the other hand, its adaptability to stressful situations may later make it resistant to the same or other types of stress, and this exposure to multiple stresses during its lifecycle in the food strain can create the possibility of its survival [7]. Indeed, *A. butzleri* strains can tolerate high levels of sodium chloride (salt: NaCl) and grow at low temperatures. In addition, they can adhere to a variety of surfaces, and all of these features may justify the presence of these bacteria in food [8, 9]. Today, there is a strong interest in developing an assortment of mathematical models for environmental issues including food, oxygen, water, pH, temperature, and effective preservatives shaping the growth, survival, and inactivation of bacteria. Previously, there were a few mathematical models to improve food durability and safety through regulating environmental factors [1]. However, little is known about the pathogenic mechanisms and the pathogenic genes of *A. butzleri*. On the other hand, determining the RM4018 genome sequence of *A. butzleri* has verified the presence of 10 pathogenic genes of cadF, *mviN*, pldA, *tlyA*, cj1349, hecB, irgA, hecA, *ciaB*, and iroE [10-12]. Nevertheless, it is not still clear whether the pathogenic factors are characterized by similar functions for their homologs in other bacterial species or not [4]. Given the importance of pathogenic genes, the present study was to isolate *A. butzleri* from environmental samples, to determine the presence of *ciaB*, contributing to host cell invasion via the secretory system, *tlyA*, and encoding hemolysin and *mviN* as an essential protein required for peptidoglycan biosynthesis, and ultimately to evaluate their survival rates and expressions under different physicochemical conditions.

Materials and Methods

Isolation and Identification of *A. butzleri* Strains

A total number of 384 samples were collected, including 238 cases from the wastewater discharged by a poultry slaughterhouse and 146 cases from the Cheshmeh Kileh River in the city of Tonekabon, northern Iran. The samples were then cultivated onto the Preston medium, enriched with supplements, and incubated at 25ºC for 24 h. Afterwards, the given samples were inoculated onto Campylobacter Selective Agar (CAMPY), enriched with defibrinated sheep blood and Campylobacter supplement. Subsequently, the plates were incubated at 25ºC for 48-72 h. The suspected *A. butzleri* colonies were also identified using Gram staining, catalase, fermentation of glucose, lead (II) acetate ($Pb(CH_3COO)_2$), nitrate (NO_3^-) reduction reaction, urease, growth in 1% glycine, increase in 3.5% NaCl, as well as resistance to nalidixic acid (NA: $C_{12}H_{12}N_2O_3$) and Cephalothin as an antibiotic [13].

Molecular Identification of *A. butzler*i

For the purpose of molecular identification, deoxyribonucleic acid (DNA) was extracted according to the cDNA synthesis kit (GeneAll extraction kit, Korea). In addition, the 16S ribosomal ribonucleic acid (rRNA) gene and the virulence genes of *mviN*, *ciaB*, and *tlyA* of *A. butzleri* were identified using their specific primers (Table 1). Each polymerase chain reaction

Table 1. Forward/reverse primer sequences for 16s RNA gene and pathogenic genes of *A. butzleri*

Name of Primer	Sequence (5'→3')	Target gene	Amplicon length	Reference
Arco1	GTCGTGCCAAGAAAAGCCA	16S rRNA	1.223 bp	(Douidah et al. 2012; Tabatabaei et al. 2014)
Arco2	GGTGTAGGATGAGACTATATA			
*Cia*B-F	TGGGCAGATGTGGATAGAGCTTGGA	*CiaB*	284 bp	
*Cia*B-R	TAGTGCTGGTCGTCCCACATAAAG			
MviN-F	TGCACTTGTTGCAAAACGGTG	*MviN*	294 bp	(Douidah et al. 2012; Tabatabaei et al. 2014)
MviN-R	TGCTGATGGAGCTTTTACGCAAGC			
TlyA-F	CAAAGTCGAAACAAAGCGACTG	*TlyA*	230 bp	
TlyA-R	TCCACCAGTGCTACTTCCTATA			

(PCR) amplification was correspondingly carried out via a 20 µl reaction mixture containing 10 µl of Master Mix (HotStartTaq Polymerase, dNTP, buffer, Mgcl), 1 µl of forward primer, 1 µl of reverse primer, 3 µl of distilled water, and 5 µl of DNA for the desired gene.

The cycling conditions were initial denaturation at 94ºC for 4 min followed by 25 cycles of denaturation at 94ºC for 1 min, annealing at 56ºC for 1 min and 72ºC for 1 min, and extension at 72ºC for 7 min. In addition, the cycling condition for *mviN* and *ciaB* gene were 94ºC for 3 min followed by 35 PCR cycles of denaturation at 94ºC for 45 s, 56ºC for 45 s, and 72ºC for 45 s, and the final extension at 72ºC for 10 min. For *tlyA*, the thermacycler instrument was set at 94ºC for 3 min for initial denaturation. Afterwards, 35 PCR cycles included 94ºC for 45 s, 55ºC for 45 s, and 72ºC for 45 s, and the final extension at 72ºC for 10 min [12-14]. Ultimately, the PCR products were subjected to electrophoresis with 1.5% agarose gel, stained with ethidium bromide (C21H20BrN3), and then photographed under a gel documentation system. Subsequently, the amplicons were purified and sent for direct sequencing to Sequetech Corporation. The pathogenic gene expression of *tlyA*, *ciaB*, and *mviN* was assessed applying the cDNA synthesis kit (GeneAll extraction kit, Korea) based on PCR and specific primers.

Effect of Various Environmental Stresses on Isolates

To this end, the identified bacteria were incubated in 25 ml of the pre-made Preston medium and then kept at 25ºC for 48 h. Then, turbidity level was assessed with the 0.5 McFarland standards. Subsequently, 1 ml of the samples was transferred to the Preston medium with various pH levels of 5.0, 6.0 (citrate buffer), 7.0, 8.0 (barbital buffer), and 9.0 (glycine buffer) and incubated at 25ºC for 24 h. The bacterial growth was additionally evaluated before and after the shocks using turbidity meter and spectrophotometry. The same procedure was further completed for the effect of different temperatures (namely, 20, 25, 30, 35, and 40ºC) [2] as well as NaCl concentrations (1%, 3%, and 5%) [1]. To evaluate the effect of the ultraviolet (UV) light exposure, the inoculates placed in empty plates were set at a distance of 34 cm, below the UV device with a wavelength of 250 nm, at 30, 60, 90, and 120 s [15]. Then, the samples were cultivated onto CAMPY under sterile conditions and kept at 25ºC for 48 h. Following the evaluation of different environmental stresses, the reverse transcription (RT)-PCR was recruited to reflect on the expression of the pathogenic genes [8].

Results and Discussion

Isolation, Identification and Occurrence of the Isolates

Totally, 58 samples were confirmed according to *A. butzleri* phenotyping. As shown in Table 2, most of the isolates belonged to springtime and the samples extracted from the poultry slaughterhouse wastewater (34.48%). However, fewer isolates were related to the winter with an occurrence rate of 5.17% in the samples obtained from the poultry slaughterhouse wastewater and no strains had been isolated from the Cheshmeh Kileh River.

Molecular Identification of Isolates

The isolates in this study were molecularly confirmed based on the 16S rRNA gene sequencing and they showed pieces 1.223 base pair (bp) (Fig. 1). Furthermore, all the three isolated strains (100%) demonstrated the presence of *ciaB* (284 bp) (Fig. 2), *mviN* (294 bp) (Fig. 3), and *tlyA* (230 bp) genes (Figure 4). According to the results obtained from the National Center for Biotechnology Information (NCBI), the first isolate belonged to *A. butzleri* strain NCTC (LT906455.1) with 93% homology. As well, the second and the third isolates were recognized as *A. butzleri* JV22 (CP040507.1) and *A. butzleri* ED-1 (AP012047.1) with 97% and 99% similarity, respectively.

Effect of Various Environmental Stresses on Isolates

The impact of various environmental stresses in the present study was assessed on the bacterial growth

Table 2. Number/frequency of *A. butzleri* strains isolated from the *Cheshmeh Kileh* River and poultry slaughterhouse wastewater.

Seasons	Sample collection	Number of isolates	Number of isolates from Cheshmeh Kileh River	Number of isolates from poultry slaughterhouse wastewater
Spring	139	25 (6.5%)	5 (8.62%)	20 (34.48%)
Summer	99	10 (2.6%)	2 (3.44%)	8 (13.79%)
Fall	89	20 (5.2%)	9 (15.51%)	11 (18.96%)
Winter	57	3 (0.7%)	-	3 (5.17%)
Total	384	58 (15.1%)	16 (27.57%)	42 (72.4%)

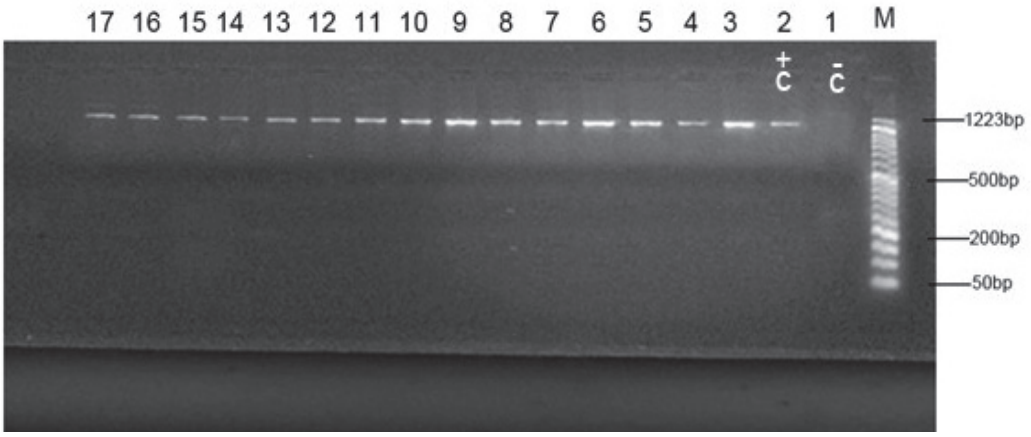

Fig. 1. Results of PCR on 1.5% agarose gel, Lane 1, negative control, Lane 2, positive control (a strain confirmed in previous studies), Lanes 3-17, samples of *A. butzleri* isolated. M, Marker (1.5-kilo bp).

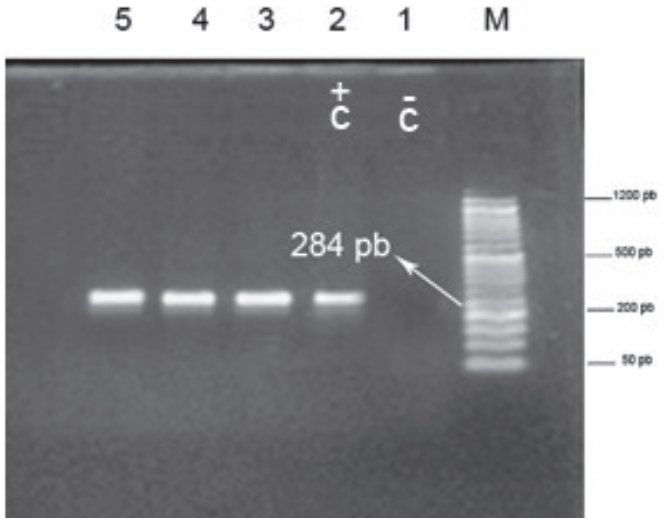

Fig. 2. Results of PCR on 1.5% agarose gel, Lane 1, negative control, Lane 2, positive control (a strain confirmed in previous studies), Lanes 3-5, one-third of samples of *A. butzleri* that contained the *ciaB* gene. M, Marker (1.5-kilo bp).

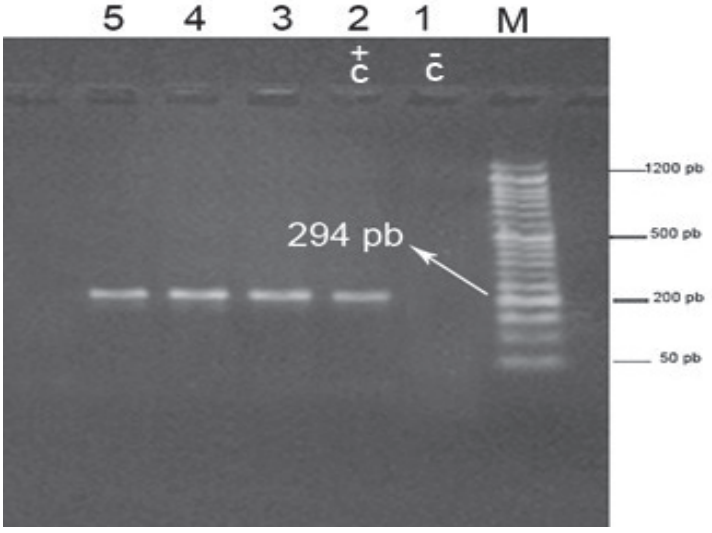

Fig. 3. Results of PCR on 1.5% agarose gel, Lane 1, negative control, Lane 2, positive control (a strain confirmed in previous studies), Lanes 3-5, one-third of samples of *A. butzleri* that contained the *mviN* gene. M, Marker (1.5-kilo bp).

rate at the time of inoculation and 24 h later. The data analysis was further performed using the SPSS Statistics software (version 22) through descriptive (frequency, frequency percentage, mean, and standard deviation (SD) and inferential (paired-samples t-test and one-way analysis of variance (ANOVA) statistics. In this resepct, the paired-samples t-test results regarding the effect of various pH levels on the bacterial growth rate before and 24 h later revealed a significant difference between the pH levels of 5.0, 7.0, 8.0, and 9.0 ($p<0.05$). In addition, the bacterial growth rate decreased 24 h following inoculation at a pH level of 5.0 but elevated at the pH levels of 7.0, 8.0, and 9.0. Moreover, the results of one-way ANOVA established a significant difference in the bacterial growth rate at the mentioned pH levels during inoculation and 24 h later ($p<0.05$). All through inoculation, the highest level of bacterial growth was related to the pH level of 7.0, whereas the lowest amount was observed at pH levels of 6.0 and 8.0. Furthermore, the lowest and the highest bacterial growth levels were associated with the pH levels of 7.0, 5.0 and 6.0, respectively, 24 h after the inoculation.

In terms of the effects of various temperatures, the paired-samples t-test results confirmed a significant rise in the bacterial growth rate during inoculation and 24 h later ($p<0.05$). On the other hand, the one-way ANOVA results indicated no significant difference between various temperatures with regard to bacterial growth rate ($p>0.05$). Meanwhile, there was a significant difference between various temperatures 24 h after the inoculation ($p<0.05$). According to the obtained results, the highest bacterial growth rates were observed at 20, 25, and 35ºC temperatures, whereas the lowest growth rate was detected at 40ºC.

Concerning the effects of various NaCl concentrations, the paired-samples t-test results demonstrated a significant decline in the bacterial growth rate during the inoculation and 24 h later in the presence of different NaCl concentrations (1, 3, and 5%) ($p<0.05$). Accordingly, there was a significant difference between various NaCl concentrations during the inoculation and 24 h later in terms of the bacterial growth rate. In this regard, the highest bacterial growth rate was related to 3% NaCl concentration during the inoculation, whereas the lowest level was observed at 1% NaCl concentration. Moreover, the highest and the lowest bacterial growth rates were related to 3 and 5% NaCl concentrations, respectively, 24 h following the inoculation.

Regarding the effects of UV light exposure time, the results indicated a decreased bacterial growth rate in all times (30, 60, and 90 s) during 24 h after the inoculation ($p<0.05$). Furthermore, the one-way ANOVA results showed no significant difference between various UV light exposure times in terms of the bacterial growth rate during 24 h following the inoculation ($p>0.05$). Moreover, 24 h after the inoculation, the highest and the lowest growth rates related to UV light exposure times were reported by 30 and 90 s, respectively.

Expression of Pathogenic Genes after Environmental Stresses

pH Levels

The RT-PCR was performed on three strains of *A. butzleri* at the pH levels of 5.0-9.0 using the specific primers of $CiaB_1$, $CiaB_2$, $mviN_1$, $mviN_2$, $tlyA_1$, and $tlyA_2$. In this regard, the proliferation of 284, 290 and 230 bp segments confirmed the expression of *ciaB*, *mviN*, and *tlyA* pathogenic genes, respectively. Moreover, their lack of proliferation approved no expression of the mentioned genes. Accordingly, all three *ciaB*, *mviN*, and *tlyA* pathogenic genes were expressed in bacterium No. 1 at all pH levels (viz. 5.0-9.0) (Fig. 5). On the other hand, the *ciaB* gene was expressed in bacterium No. 2 only at the pH levels of 7.0 and 8.0. The *mviN* gene was further expressed only at the pH level of 7.0, whereas the *tlyA* gene was expressed just at the pH levels of 7.0 and 8.0 (Fig. 6). In bacterium No. 3, the *ciaB* and *mviN* genes were expressed only at the pH levels of 6.0, 7.0 and 8.0, and the *tlyA* gene was expressed merely at the pH levels of 6.0 and 7.0 (Fig. 7).

Temperature

The RT-PCR was practiced on three strains of *A. butzleri* at different temperatures of 35, 30, 25, 20, and 40ºC using the specific primers of $CiaB_1$, $CiaB_2$, $mviN_1$, $mviN_2$, $tlyA_1$, and $tlyA_2$. In this study, the *ciaB* gene was expressed only at 35, 30, and 20ºC in 1-3 bacteria, respectively. Moreover, the *mviN* gene was expressed at 40, 35, 30, and 20ºC, whereas the *tlyA* gene was expressed only at 35, 30, and 25ºC (Figs 8-10).

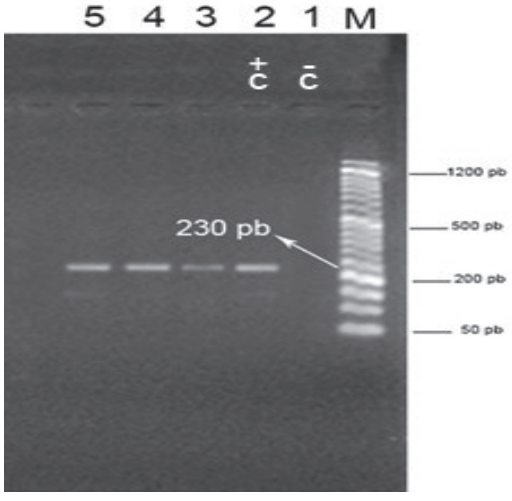

Fig. 4. Results of PCR on 1.5% agarose gel, Lane 1, negative control, Lane 2, positive control (a strain confirmed in previous studies), Lanes 3-5, one-third of samples of *A. butzleri* that contained the *tlyA* gene. M, Marker (1.5-kilo bp).

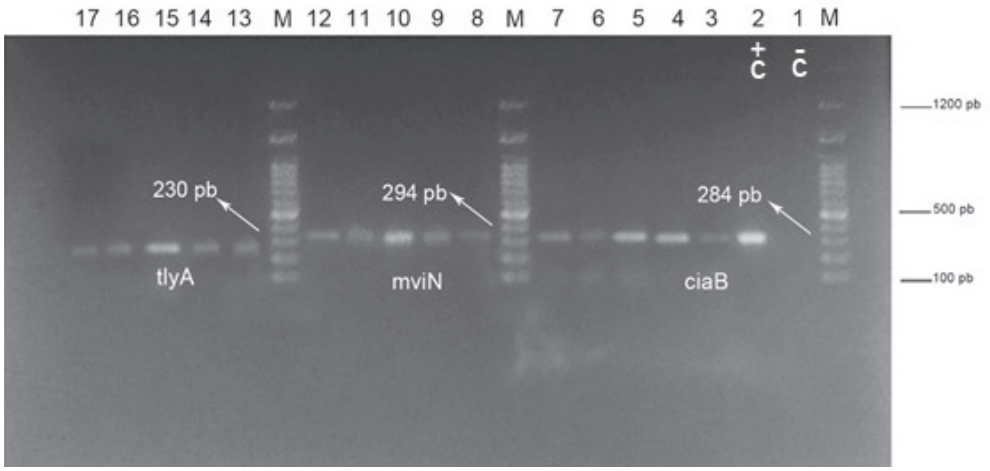

Fig. 5. Results of RT-PCR on 1.5% agarose gel following the effect of different pH levels in sample No. 1. From the right side, Lane 1, negative control, Lane 2, positive control, Lanes 3-7, the *ciaB* gene at pH levels of 5.0-9.0, respectively, Lanes 8-12, the *mviN* gene at pH levels of 5.0-9.0, respectively, Lanes 13-17, the *tlyA* gene at pH levels of 5.0-9.0, respectively. M, Marker (1-kilo bp).

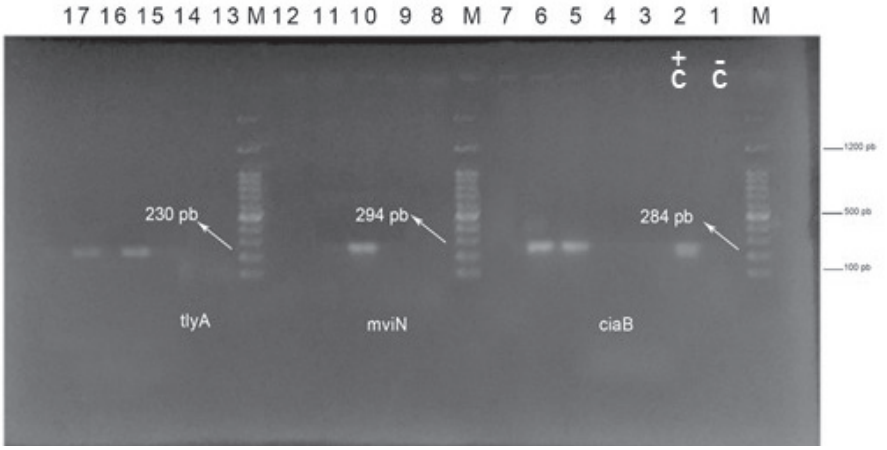

Fig. 6. Results of RT-PCR on 1.5% agarose gel following the effect of different pH levels in sample No. 2. From the right side Lane 1, negative control, Lane 2, positive control, Lanes 3-7, the *ciaB* gene at pH levels of 5.0-9.0, respectively, Lanes 8-12, the *mviN* gene at pH levels of 5.0-9.0, respectively, Lanes 13-17, the *tlyA* gene at pH levels of 5.0-9.0, respectively. M, Marker (1-kilo bp).

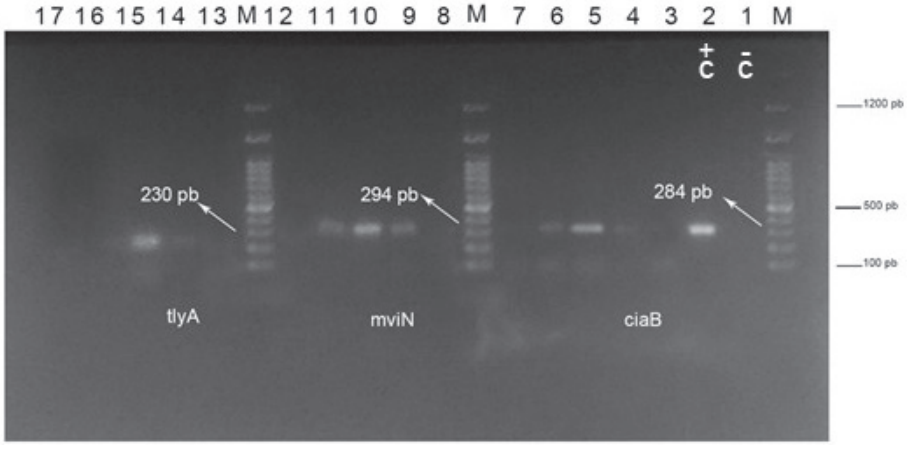

Fig. 7. Results of RT-PCR on 1.5% agarose gel following the effect of different pH levels in sample No. 3. From the right side Lane 1, negative control, Lane 2, positive control, Lanes 3-7, the *ciaB* gene at pH levels of 5.0-9.0, respectively, Lanes 8-12, the *mviN* gene at pH levels of 5.0-9.0, respectively, Lanes 13-17, the *tlyA* gene at pH levels of 5.0-9.0, respectively. M, Marker (1-kilo bp).

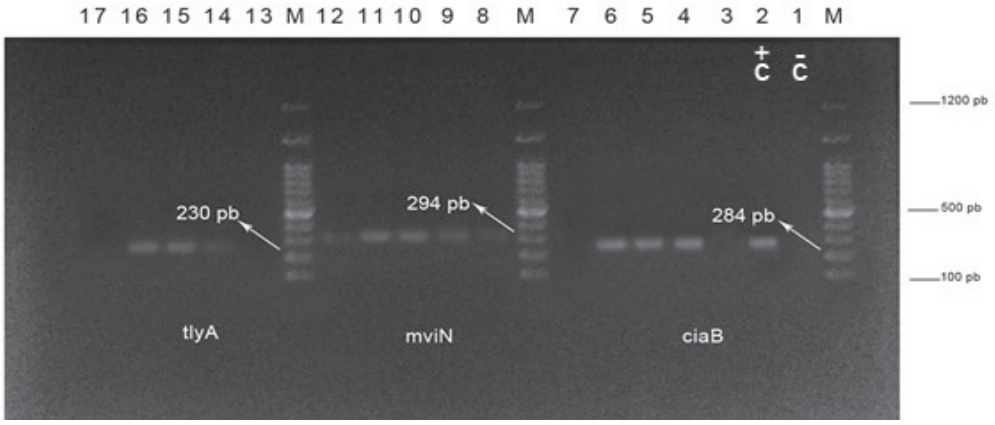

Fig. 8. Results of RT-PCR on 1.5% agarose gel following the effect of different temperatures in sample No. 1. From the right side, Lane 1, negative control, Lane 2, positive control, Lanes 3-7, the *ciaB* gene at different temperatures of 20, 25, 30, 35, and 40°C, respectively, Lanes 8-12, the *mviN* gene at different temperatures of 20, 25, 30, 35, and 40°C, respectively, Lanes 13-17, the *tlyA* gene at different temperatures of 20, 25, 30, 35, and 40°C, respectively. M, Marker (1-kilo bp).

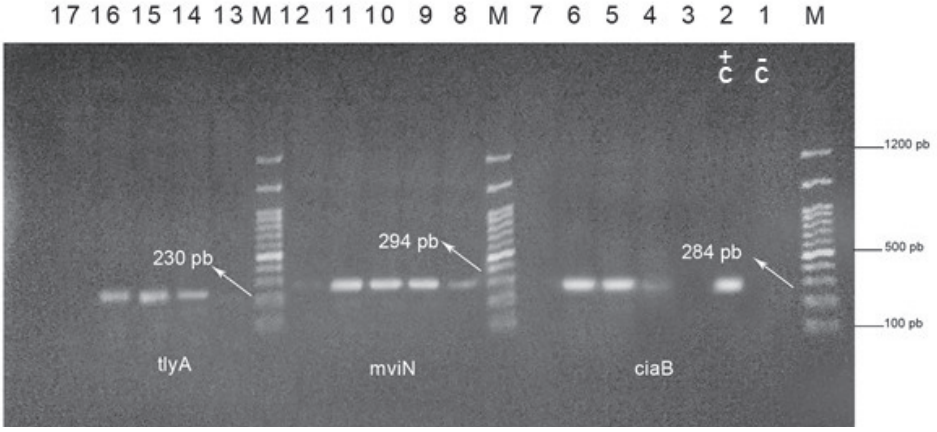

Fig. 9. Results of RT-PCR on 1.5% agarose gel following the effect of different temperatures in sample No. 2. From the right side, Lane 1, negative control, Lane 2, positive control, Lanes 3-7, the *ciaB* gene at different temperatures of 20, 25, 30, 35, and 40°C, respectively, Lanes 8-12, the *mviN* gene at different temperatures of 20, 25, 30, 35, and 40°C, respectively, Lanes 13-17, the *tlyA* gene at different temperatures of 20, 25, 30, 35, and 40°C, respectively. M, Marker (1-kilo bp).

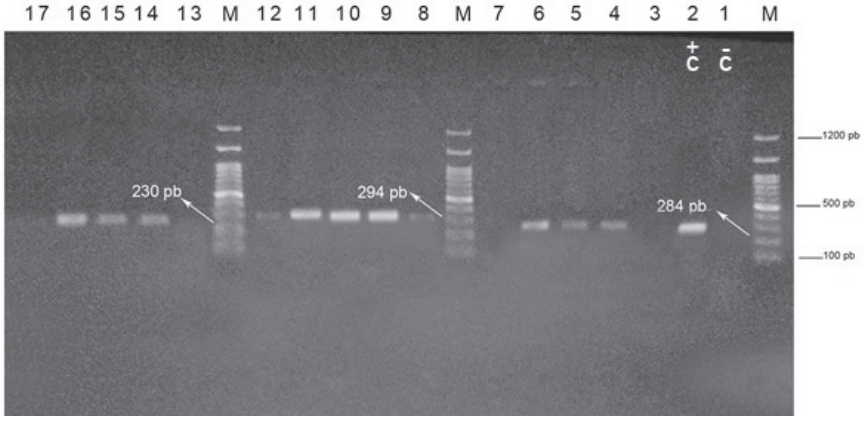

Fig. 10. Results of RT-PCR on 1.5% agarose gel following the effect of different temperatures in sample No. 3. From the right side, Lane 1, negative control, Lane 2, positive control, Lanes 3-7, the *ciaB* gene at different temperatures of 20, 25, 30, 35, and 40°C, respectively, Lanes 8-12, the *mviN* gene at different temperatures of 20, 25, 30, 35, and 40°C, respectively, Lanes 13-17, the *tlyA* gene at different temperatures of 20, 25, 30, 35, and 40°C, respectively. M, Marker (1-kilo bp).

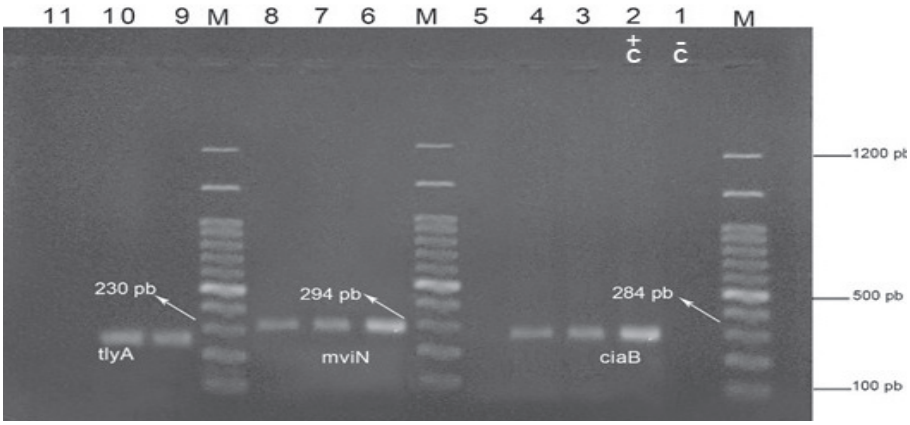

Fig. 11. Results of RT-PCR on 1.5% agarose gel following the effect of different NaCl concentrations in sample No. 1. From the right side, Lane 1, negative control, Lane 2, positive control, Lanes 3-5, the *ciaB* gene at 1, 3, and 5% NaCl concentrations, respectively, Lanes 6-8, the *mviN* gene at 1, 3, and 5% NaCl concentrations, respectively, Lanes 9-11, the *tlyA* gene at 1, 3, and 5% NaCl concentrations, respectively. M, Marker (1-kilo bp).

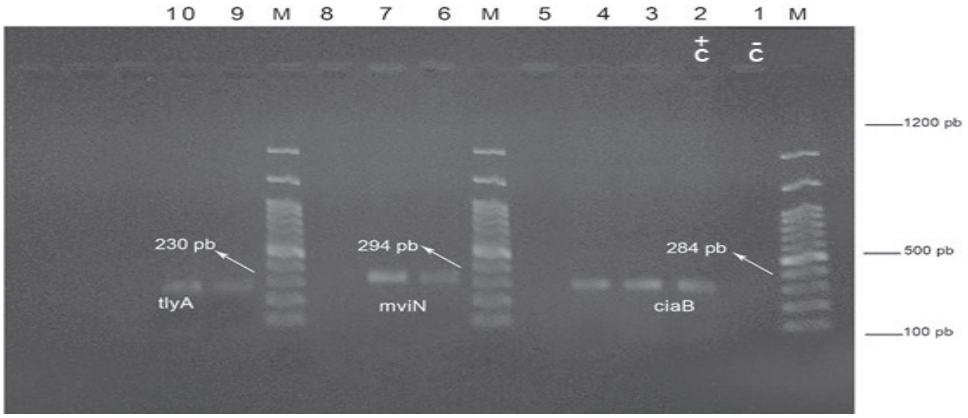

Fig. 12. Results of RT-PCR on 1.5% agarose gel following the effect of different NaCl concentrations in sample No. 2. From the right side, Lane 1, negative control, Lane 2, positive control, Lanes 3-5, the *ciaB* gene, Lanes 6-8, the *mviN* gene, Lanes 9-11, the *tlyA* gene. M, Marker (1-kilo bp).

NaCl Concentrations

The RT-PCR was performed on three strains of *A. butzleri* at different NaCl concentrations (viz. 1, 3, and 5%) using the specific primers of $CiaB_1$, $CiaB_2$, $mviN_1$, $mviN_2$, $tlyA_1$, and $tlyA_2$. The *ciaB* gene was also expressed in bacteria No. 1 and 3 at NaCl concentrations of 1 and 3% but it was not expressed at 5% concentration. On the other hand, the *mviN* gene was expressed at all NaCl concentrations and the *tlyA* gene was simply expressed at 1 and 3% concentrations (Figs 11 and 12). In bacterium no. 2, all three *tlyA*, *ciaB*, and *mviN* pathogenic genes were expressed at 1 and 3% NaCl concentrations and none of them was expressed at 5% concentration (Fig. 13).

UV Light Exposure Time

The RT-PCR was done at different UV light exposure times using the specific primers. All three *tlyA*, *ciaB*, and *mviN* pathogenic genes were accordingly expressed only at 30 and 60 s UV light exposure times in bacterium No. 1 (Fig. 14). Meanwhile, the *ciaB* and *mviN* genes were expressed only at 30 and 60 s UV light exposure times in bacterium no. 2 and the *tlyA* gene was expressed only at 30 s exposure time (Fig. 15). In bacterium no. 3, the *ciaB* and *tlyA* genes were also expressed only at 30 and 60 s UV light exposure times, whereas the *mviN* gene was expressed at all 30, 60, and 90 s exposure times (Fig. 16).

To date, no adequate information has been reported about *A. butzleri* pathogenic mechanisms (e.g., adhesion, invasion, or toxicity). The global prevalence of *A. butzleri* infection is also unclear since no conventional diagnostics methods have been implemented for the bacterium concerned. There is no doubt that *A. butzleri* strains have pathogenic effects on humans. Nevertheless, further clinical observations are required to determine the interference of *A. butzleri* strains in human infections [16]. The association of

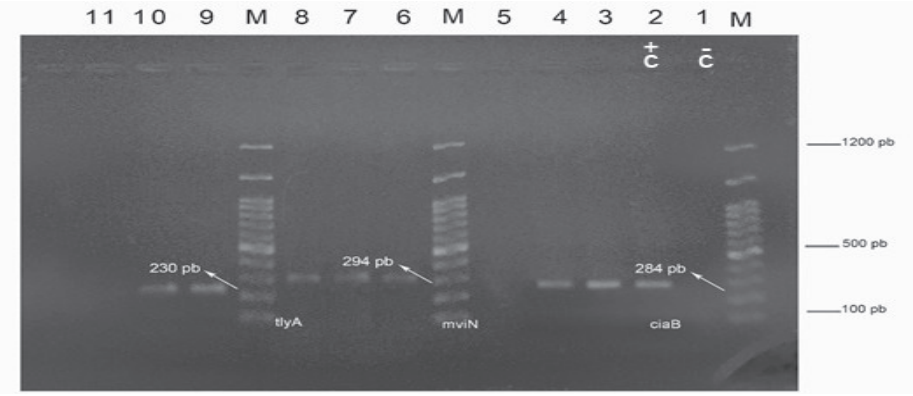

Fig. 13. Results of RT-PCR on 1.5% agarose gel following the effect of different NaCl concentrations in sample No. 3. From the right side, Lane 1, negative control, Lane 2, positive control, Lanes 3-5, the *ciaB* gene, Lanes 6-8, the *mviN* gene, Lanes 9-11, the *tlyA* gene, M, Marker (1-kilo bp).

A. butzleri species with human pathogenesis, as well as their discovery and spread in food and water, has also confirmed the necessity of further studies on the survival rates of this microorganism, as well as the search for new therapies to control and eliminate this bacterium. The significant survival of *A. butzleri* on the plastic surface can be similarly a factor in the transmission of this bacterium to humans during food processing [17].

Most studies on the prevalence of *A. butzleri* are related to poultry feed, which has the highest rate, followed by pork products and raw milk [18, 19]. In the case of poultry, there are still debates about the sources of contamination, as some researchers believe that the environment of slaughterhouses, not their wastes, has been the main source due to no ability to isolate *A. butzleri* from the feces. However, some researchers have established that the bacteria could be living in the intestines of poultry, demonstrating that factors such as animals, samples, and methods applied have affected the evaluation and the identification of the prevalence rate of the given bacterium [9]. The high resistance of *A. butzleri*, along with its ability to survive and grow in aerobic conditions, has also increased its potential as a food- and water-borne pathogen. On the other hand, its adaptability to stressful situations may later make it resistant to this or other types of stress. Therefore, exposure to various stresses during a lifecycle creates its survival rate in the food strain [7].

Since most bacteria deal with environmental stresses during long periods of their lives, more information and findings in this regard as well as associated responses are required to understand their physiology. This can further reduce the levels of contamination in environmental samples such as poultry slaughterhouse wastewater to prevent the spread of bacteria to the environment and its transmission to humans. It also leads to the development of new methods for environmental health, veterinary medicine, treatment of diseases, as well as introduction of new antimicrobial agents. Therefore, studying responses to stresses will be a new topic in biological and applied research.

In the present study, poultry slaughterhouse wastewater, culture techniques, and the PCR/RT-PCR

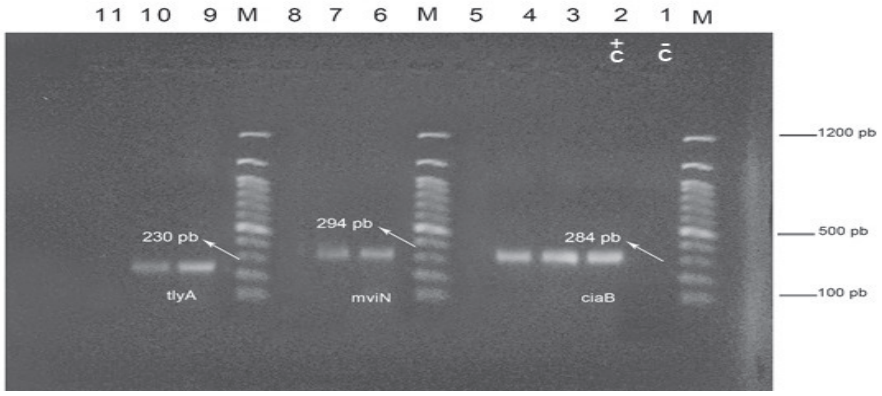

Fig. 14. Results of RT-PCR on 1.5% agarose gel following the effect of 30, 60, and 90 s UV light exposure times in sample No. 1. From the right side, Lane 1, negative control, Lane 2, positive control, Lanes 3-5, the *ciaB* gene at 30, 60, and 90 s UV light exposure times, respectively, Lanes 6-8, the *mviN* gene at 30, 60, and 90 s UV light exposure times, respectively, Lanes 9-11, the *tlyA* gene at 30, 60, and 90 s UV light exposure times, respectively. M, Marker (1-kilo bp).

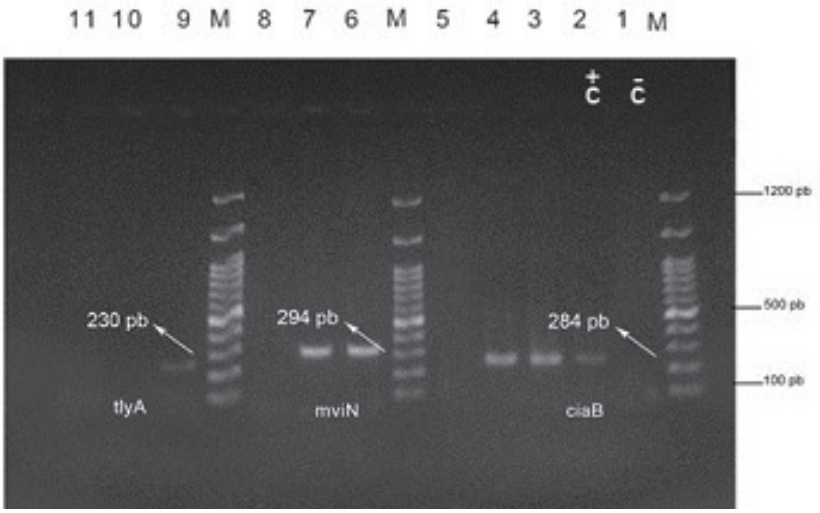

Fig. 15. Results of RT-PCR on 1.5% agarose gel following the effect of 30, 60, and 90 s UV light exposure times in sample No. 2. From the right side, Lane 1, negative control, Lane 2, positive control, Lanes 3-5, the *ciaB* gene at 30, 60, and 90 s UV light exposure times, respectively, Lanes 6-8, the *mviN* gene at 30, 60, and 90 s UV light exposure times, respectively, Lanes 9-11, the *tlyA* gene at 30, 60, and 90 s UV light exposure times, respectively. M, Marker (1-kilo bp).

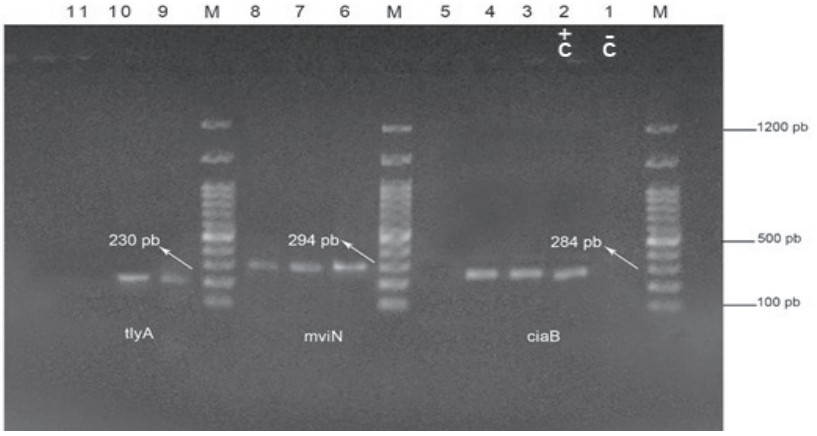

Fig. 16. Results of RT-PCR on 1.5% agarose gel following the effect of 30, 60, and 90 s UV light exposure times in sample No. 3. From the right side, Lane 1, negative control, Lane 2, positive control, Lane 3-5, the *ciaB* gene at 30, 60, and 90 s UV light exposure times, respectively, Lanes 6-8, the *mviN* gene at 30, 60, and 90 s UV light exposure times, respectively, Lanes 9-11, the *tlyA* gene at 30, 60, and 90 s UV light exposure times, respectively. M, Marker (1-kilo bp).

were applied to isolate and identify *A. butzleri*. Overall, 58 *A. butzleri* samples were identified among 384 cases. Similarly, Ghane et al. (2012) had identified 15 strains of *A. butzleri* using the same methods [20]. The samples could be thus affected by environmental factors; therefore, a change of seasons was taken into account and the prevalence of this bacterium in different seasons of the year was examined. In the springtime, five strains of *A. butzleri* with a frequency of 8.62% were isolated from the Cheshmeh Kileh River, whereas 20 strains with a frequency of 34.48% were isolated from wastewater discharged by the poultry slaughterhouse.

In the summer, two strains of *A. butzleri* with a frequency of 3.44% were also isolated from the Cheshmeh Kileh River, but eight strains with a frequency of 13.79% were isolated from the poultry slaughterhouse wastewater. In fall, nine strains of *A. butzleri* with a frequency of 15.51% were similarly isolated from the Cheshmeh Kileh River, whereas 11 strains with a frequency of 18.96% were isolated from the poultry slaughterhouse wastewater. In the wintertime, three strains of *A. butzleri* with a frequency of 5.17% were also isolated from the Cheshmeh Kileh River, while no strains were isolated from the poultry slaughterhouse wastewater. As such, the results of the present study showed that different seasons were of utmost importance in isolating this bacterium and the highest rate of isolation of *A. butzleri* with a frequency of 43.1% had occurred in the springtime. Similar results had been correspondingly reported

by Collado et al. (2010) [21], Ghorbani et al. (2014) [22], and Ghaju Shrestha et al. (2019) [23].

As some bacteria may lose their pathogenicity, after the induction of some stresses, or at least their pathogenicity may significantly reduce, the study findings could be utilized to evaluate various effects of environmental stresses on the growth rate and expression of these bacteria and to find proper solutions to control their transmission. In addition, fundamental research would be carried out on some topics, not sufficiently covered.

In the present research, various physicochemical conditions were further applied to three strains of *A. butzleri*, which confirmed the presence of *mviN*, *ciaB* and *tlyA* pathogenic genes based on the PCR/RT-PCR. According to the results of the present study, the lowest bacterial growth rate after 24 h was related to the pH levels of 5.0 and 6.0, which was consistent with the findings obtained by Lee and Choi (2013) [15] and Young Park and Do Ha (2015) [1]. The lowest bacterial growth rate was related to 5% NaCl concentration and 90 s UV light exposure time. In this line, Young Park and Do Ha (2012) had further reported a descending trend in the growth rate of *A. butzleri* at ≥4% NaCl concentration.

The main objective of the present study was to discontinue the expression of *tlyA*, *ciaB*, and *mviN* pathogenic genes of *A. butzleri* by applying various stresses. As mentioned before, the RT-PCR was applied to evaluate the expression/lack of expression of the mentioned genes. Overall, the study results showed that the *ciaB* gene expression was prevented at the pH levels of 5.0, 6.0, and 9.0, at 20 and 40ºC, at 5% NaCl concentration, and at 90 s UV light exposure time. In addition, the *mviN* gene expression was discontinued at the pH levels of 5.0, 6.0, 8.0, and 9.0 at 5% NaCl concentration, at oxygen concentration of 180 rpm, and at 90 s UV light exposure time. Moreover, the *tlyA* gene expression was prevented at the pH levels of 5.0, 6.0, 8.0, and 9.0, at 20 and 40ºC, at 5% NaCl concentration, and at 60 and 90 s UV light exposure times. As no research has so far been conducted, to the best of authors' knowledge, on the effect of environmental stresses on the expression of *A. butzleri* pathogenic genes this study could be the first attempt in biological and applied research on bacteria.

Conclusion

According to the results of the present study, *A. butzleri* was found in the poultry slaughterhouse wastewater and the Cheshmeh Kileh River, and the highest amount of the bacteria isolated was related to the wastewater in springtime. The results also indicated the presence of all *tlyA*, *ciaB*, and *mviN* pathogenic genes in all *A. butzleri* strains. These data suggested that *A. butzleri* might endanger human health because the presence of pathogenic genes could be a potential risk factor for the bacterium. Invasive diseases caused by *A. butzleri* could be thus a serious threat to individuals with impaired immune systems.

The results of the effect of various environmental stresses on the expression of pathogenic genes correspondingly demonstrated that the expression of *tlyA*, *ciaB*, and *mviN* genes discontinued at the pH levels of 5.0, 6.0, and 9.0, at 5% NaCl concentration, at oxygen concentration of 180 rpm, and 90 s UV light exposure time. Furthermore, the *tlyA* and *ciaB* gene expression stopped at 20 and 40ºC, while temperature had no impact on the expression of the *mviN* gene. The study also revealed that the expression of the pathogenic genes was prevented while the bacterial growth rate increased due to the effect of temperature. The study findings could be a proper solution to discontinue the transmission of the bacterium through poultry slaughterhouse wastewater and rivers. In addition, they could be applied to improve microbiological quality of food. Furthermore, an effective step could be taken to determine the proper treatment of diseases induced by *A. butzleri*.

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Conflict of Interest

The authors declare no conflict of interest.

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