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Effect of Stress-Adaptation on Antibiotic Sensitivity Profiles of *Campylobacter jejuni*

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ABSTRACT

Campylobacter jejuni is one of the leading causes of human gastroenteritis. *Campylobacter jejuni* requires special conditions and media in the laboratory for its growth. In nature, however, this organism is able to survive in very diverse and hostile environments and produce disease in humans and animals. The different mechanisms by which *C. jejuni* survives stressful conditions in the environment still remain unclear. Stress-adaptation may be one of the factors helping this organism to survive stresses. Some *C. jejuni* strains have been found to have increased antibiotic resistance in last several years. To determine the effect of acid adaptation on the antibiotic sensitivity profile of *C. jejuni*, 4 different isolates of *C. jejuni* (a human isolate and 3 poultry isolates) were exposed to an acid pH of 5.5 and then re-challenged with different stresses. The antibiotic sensitivity profiles of *C. jejuni* after stress-adaptation were compared with antibiotic sensitivity profiles of non-stressed *C. jejuni* using the Kirby Bauer agar disc diffusion assay. The antibiotic sensitivity profiles of the *C. jejuni* isolates used in this study were found to change when the acid-adapted bacteria were subjected to further stresses such as an acidic pH of 4.5, aerobic atmosphere and starvation. In the majority of the cases, antibiotic-resistant *C. jejuni* isolates were found to be more sensitive to antibiotics after stress-adaptation, but in a few cases *C. jejuni* showed increased resistance. These results indicate that increasing various stresses in a sequential pattern may, in some cases, reduce antibiotic resistance of *C. jejuni* isolates.

Keywords: *Campylobacter jejuni*; Stress; Stress-Adaptation; Antibiotic Resistance; Antibiotics

1. Introduction

Campylobacter jejuni is among the leading causes of foodborne bacterial diarrheal disease with approximately 850,000 cases per year in the United States with an associated economic loss estimated to be 1.5 billion dollars [1]. Other possible sequelae of *C. jejuni* infections include Guillain-Barré syndrome (GBS), reactive arthritis, and irritable bowel syndrome [2]. Sources of infection for *C. jejuni* are primarily associated with poultry and poultry products since *C. jejuni* are often found as commensals in large numbers in the gastrointestinal tracts of birds [3,4]. *C. jejuni* is predominantly found to cause gastrointestinal enteritis in humans and even a very low dose, as few as 500 organisms, can cause infection [5]. The incubation period of foodborne campylobacteriosis is usually 4 - 5 days but can range from 1 - 10 days [6]. The symptoms associated with this disease include fever, diarrhea, headache, abdominal pain, myalgia, vomiting and blood in feces [7]. In majority of the infections caused by *C. jejuni*, the use of antimicrobials is not necessary as infected persons usually recover within 5 - 8 days [5]. But in some of the cases, where the infections are not found to subside within 3 - 4 days, antibiotic therapy may be indicated. Erythromycin is the drug of choice, but others such as ciprofloxacin, doxycycline and tetracycline are also used in the treatment of *C. jejuni* infections. Recently, increases in the antibiotic resistance patterns of *C. jejuni* against antibiotics such as ciprofloxacin, tetracycline and erythromycin have been found in *C. jejuni* isolates from food and water sources [8,9]. Presence of such resistant strains in the food chain has raised concerns over the antibiotic treatment of *Campylobacter* infections.

Considering the highly fastidious growth requirements of *C. jejuni*, and the lack of genetic survival mechanisms, the ability of *C. jejuni* to survive outside the host and cause foodborne illness is perplexing. Research shows that some bacterial foodborne pathogens are capable of surviving many of the control measures employed in the food industry by a mechanism known as ***adaptive tolerance response***. Exposure of bacteria to stressful environment induces a response called as the adaptive toler-

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ance response (ATR) which helps the bacteria to survive further homologous or heterologous stresses [10,11]. *C. jejuni* has been found to induce an adaptive tolerance response when exposed to acid or aerobic conditions [12, 13], but research involving effects of stress-adaptation on antibiotic sensitivity profiles after *C. jejuni* is exposed to secondary stresses is limited. Understanding stress response on a global level may give insight into how this fastidious organism survives outside the host. When bacteria are exposed to environmental stresses they may undergo phenotypic and genotypic changes to enhance their survival in the stressful environment [14]. These changes may give rise to cross-protection when these bacteria are exposed to secondary stresses which may also subsequently change their antibiotic resistance profiles [15]. Exposure of foodborne pathogens such as *E. coli*, *Salmonella* Typhimurium and *Staphylococcus* to sublethal food preservation stresses was found to change their antibiotic sensitivity profiles [16]. By studying the effects of an adaptive tolerance response on the antibiotic sensitivity profiles of acid-adapted *C. jejuni* after exposure to various secondary stresses could provide information on the role of stress and stress-adaptation on antibiotic resistance. These results should help in developing better control strategies to reduce/eliminate *C. jejuni* in processing environments. The aim of this research was to determine whether the ATR induced by different isolates of *C. jejuni* on adaptation to a mild acid pH had any effects on the antibiotic sensitivity profiles after they were subjected to secondary stresses including acid, starvation and exposure to oxygen.

2. Materials and Methods

2.1. Bacterial Isolates and Growth Conditions

Four *C. jejuni* isolates were selected for the present study including a human isolate, 81 - 176, known to cause the disease in human volunteers and three poultry isolates. The human isolate 81 - 176 was donated by Dr. Michael Johnson, University of Arkansas, Fayetteville, AR. The poultry isolates used were PRCC 3 (pre-chilled chicken carcass), POCC 13 (post-chilled chicken carcass) and RECC 3 (retail chicken carcass), which were obtained and isolated in our laboratory from different stages of poultry processing. Each isolate killed over 92% of HeLa cells when previously tested in an in vitro cytotoxicity assay [17]. The isolates were stored at −80˚C in *Campylobacter* enrichment (CE) broth (Acumedia®) supplemented with glycerol and sub-cultured prior to the stress experiments. Frozen stock cultures were passed twice on *Campylobacter* blood agar plates and then inoculated into CE broth and incubated in a micro-aerobic atmosphere consisting of 5% oxygen, 10% carbon dioxide and 85% nitrogen at 42˚C for 18 h to obtain the early stationary phase cultures for the experiments.

2.2. Acid-Adaptation of *C. jejuni* and Exposure to Secondary Stresses

Early stationary phase (18 h) cultures in *Campylobacter* enrichment (CE) broth were divided into two portions and centrifuged at 8000 × g for 5 min and subsequently re-suspended either in acid broth (pH 5.5) to obtain acid-adapted cells or in CE broth to obtain non-stressed cells. Acid broth was prepared by adding hydrochloric acid (HCl) directly to the CE broth. After an adaptation time of 2 h, the acid-adapted culture was divided into three portions, centrifuged and exposed to the following stresses for a period of 2 h: 1) acid stress (pH 4.5), by re-suspending in CE broth with a pH of 4.5 and incubating in microaerobic atmosphere; 2) acid and aerobic stress, by re-suspending in CE broth with a pH of 4.5 and incubating in aerobic atmosphere; 3) starvation stress by re-suspending cells in phosphate buffered saline (PBS) with a pH of 7.2. The non-stressed cultures of each isolate were centrifuged and re-suspended again in CE broth to form the control group with no stress. The experimental design is given in the form of a flow chart in **Figure 1**.

2.3. Determination of Antibiotic Sensitivity of *C. jejuni* Using Disc Diffusion Assay

The acid-adapted cultures exposed to different secondary stresses and the non-stressed cultures of *C. jejuni* were used to determine antibiotic sensitivity by the Kirby Bauer agar disc diffusion assay as described by the Clinical and Laboratory Standards Institute (CLSI) using Mueller-Hinton (MH) agar (Difco®) plates. Briefly, the agar disc diffusion assay was performed as follows. Prior to performing the assay, all the cultures were centrifuged and re-suspended in normal saline and the concentration of the cultures were determined by serial dilution and plating on CE blood agar plates as well as MH agar plates. For the assay, 100 µl of cell suspensions from each of the cultures were inoculated on to MH agar plates. Three to four antibiotic discs were evenly placed on the inoculated plates. Plates were incubated at 37˚C for 42 - 48 h under microaerobic conditions. *Escherichia coli* ATCC 25922, with known antimicrobial susceptibility, was used as the positive control for each of the antibiotics used. The antibiotic discs (BBLTM Sensi-DiscTM, Becton Dickinson) included in this study were ampicillin, 10 µg; chloramphenicol, 30 µg; ciprofloxacin, 5 µg; clindamycin, 2 µg; erythromycin, 15 µg; gentamicin, 10 µg; kanamycin, 30 µg; nalidixic acid, 30 µg; streptomycin 10 µg; tetracycline, 30 µg; and vancomycin, 30 µg. The antimicrobial inhibition zones were measured and interpreted as sensitive (S), intermediate (I) or resistant (R) according to CLSI standards and Huysmans and

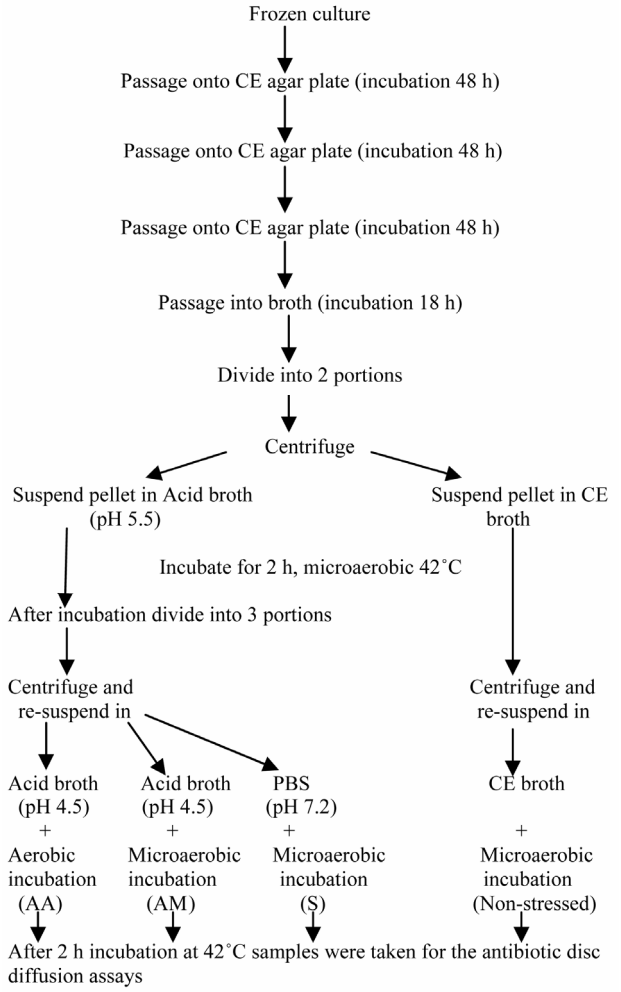

Figure 1. Flow chart of acid-adaptation and exposure of *C. jejuni* cultures to different secondary stresses.

Turnidge, 1997 [18,19]. The measurement criteria used for measuring the antimicrobial inhibition zones for the different antibiotics used in this study are shown in **Table 1**.

2.4. Statistical Analysis

The experiments were conducted in three independent replicates and the mean antibiotic inhibition zones were calculated using the JMP statistical software package 9.0.2 and the means were compared using a student's t-test. The results were considered statistically significant with p-values reported at $p < 0.05$.

3. Results

For each *C. jejuni* isolate antibiotic inhibition zones to the various antibiotics used were measured and recorded. Acid-adaptation and exposure to secondary stresses produced changes in the antibiotic sensitivity profiles of the human *C. jejuni* isolate, 81 - 176, for the antibiotics gentamicin, kanamycin, chloramphenicol and tetracycline compared to the control group with no stress (**Table 2**). All the stresses (starvation, acid and acid + aerobic exposure) changed the antibiotic sensitivity profiles of 81 - 176 for gentamicin and kanamycin from resistant and intermediate resistance to sensitive. However, for chloramphenicol and tetracycline the profiles were changed to sensitive from intermediate resistance and resistant in response to starvation and acid + aerobic exposure, respectively. Only with one antibiotic, nalidixic acid, did the sensitivity profile of 81 - 176 change to resistant from sensitive after starvation stress. The other two stresses did not produce any changes compared to the non-stressed *C. jejuni* 81 - 176.

For the *C. jejuni* poultry isolate from pre-processed chicken carcass (PRCC 3), the antibiotic sensitivity profiles were changed with 5 of the 11 antibiotics used in the study compared to the control group after acid-adapted *C. jejuni* were given the secondary stresses of either acid or starvation or acid + aerobic exposure (**Table 3**). The antibiotic sensitivity profile of PRCC 3 to ampicillin changed from sensitive to resistant with acid + aerobic exposure, whereas the acid stress produced resistance to gentamicin and intermediate resistance to kanamycin. Starvation stress was found to produce sensitive populations of PRCC 3 to chloramphenicol, whereas both starvation and acid stresses changed the antibiotic sensitivity profile for nalidixic acid from resistant to sensitive.

Similarly, for the *C. jejuni* poultry isolate from post-processed chicken carcass (POCC 13), acid-adaptation and exposure to secondary stresses produced changes in the antibiotic sensitivity profiles for 5 of the 11 antibiotics

Table 1. Disc diffusion criteria used in this study. The antimicrobial inhibition zones for the 11 antibiotics used in this study were measured and recorded according to CLSI standards and Huysmans and Turnidge, 1997 [18,19].

Antibiotics used	Zone diameter measured (mm)		
	Sensitive	Intermediate	Resistant
Ampicillin, 10 µg	≥ 10	N/A	≤ 9
Chloramphenicol, 30 µg	≥ 23	12 - 22	≤ 11
Clindamycin, 2 µg	≥ 18	16 - 17	≤ 15
Ciprofloxacin, 5 µg	≥ 24	19 - 23	≤ 18
Erythromycin, 15 µg	≥ 19	16 - 18	≤ 15
Gentamicin, 10 µg	≥ 23	N/A	≤ 22
Kanamycin, 30 µg	≥ 18	14 - 17	≤ 13
Nalidixic acid, 30 µg	≥ 15	N/A	≤ 14
Streptomycin, 10 µg	≥ 15	12 - 14	≤ 11
Tetracycline, 30 µg	≥ 33	16 - 32	≤ 15
Vancomycin, 30 µg	-	-	-

Table 2. Antibiotic profiles of *C. jejuni* human isolate 81 - 176 (S: sensitive, I: intermediate or R: resistant) after acid-adaptation and exposure to secondary stresses. The profiles were compared with the control group with no stress exposure.

Antibiotics used	Conc. of antibiotics (in µg)	Non-stressed	Acid-adapted *C. jejuni* 81 - 176 exposed to different secondary stresses		
			Starvation + Microaerobic	Acid + Microaerobic	Acid + aerobic
Ampicillin	10 µg	S	S	S	S
Chloramphenicol	30 µg	I	**S**	I	I
Clindamycin	2 µg	S	S	S	S
Ciprofloxacin	5 µg	S	S	S	S
Erythromycin	15 µg	S	S	S	S
Gentamicin	10 µg	R	**S**	**S**	**S**
Kanamycin	30 µg	I	**S**	**S**	**S**
Nalidixic acid	30 µg	S	**R**	S	S
Streptomycin	10 µg	S	S	S	S
Tetracycline	30 µg	R	R	R	**I**
Vancomycin	30 µg	R	R	R	R

Table 3. Antibiotic profiles of *C. jejuni* poultry isolate PRCC 3 (S: sensitive, I: intermediate or R: resistant) after acid-adaptation and exposure to secondary stresses. The profiles were compared with the control group with no stress exposure.

Antibiotics used	Conc. of antibiotics (in µg)	Non-stressed	Acid-adapted *C. jejuni* PRCC 3 exposed to different secondary stresses		
			Starvation + Microaerobic	Acid + Microaerobic	Acid + aerobic
Ampicillin	10 µg	S	S	S	**R**
Chloramphenicol	30 µg	I	**S**	I	I
Clindamycin	2 µg	S	S	S	S
Ciprofloxacin	5 µg	S	S	S	S
Erythromycin	15 µg	S	S	S	S
Gentamicin	10 µg	S	S	**R**	S
Kanamycin	30 µg	S	S	**I**	S
Nalidixic acid	30 µg	R	**S**	**S**	R
Streptomycin	10 µg	S	S	S	S
Tetracycline	30 µg	R	R	R	R
Vancomycin	30 µg	R	R	R	R

used in the study compared to the control group (**Table 4**). All stresses were found to change the resistant profile of non-stressed POCC 13 to sensitive with the antibiotic kanamycin. For the antibiotic nalidixic acid the profile was changed from resistant to sensitive with acid stress, whereas both acid and starvation stresses changed the profile from intermediate resistance to sensitive for streptomycin. Acid + aerobic stress changed the sensitive profile of POCC 13 towards gentamicin to resistant. The ciprofloxacin profile changed from sensitive to intermediate resistance with starvation and to resistant with acid + aerobic exposure.

The antibiotic sensitivity profiles for RECC 3, the *C. jejuni* poultry isolate from retail chicken carcass, changed with 7 of the 11 antibiotics used in this study compared to the control group after acid-adaptation and exposure to secondary stresses (**Table 5**). Exposure to the secondary stress of starvation changed the antibiotic sensitivity profiles of non-stressed RECC 3 from resistant to sensitive for ampicillin and nalidixic acid and to intermediate resistance with ciprofloxacin. For the antibiotics gentamicin and clindamycin, the secondary stresses of acid and acid + aerobic exposure changed the profile from sensitive to resistant, whereas for starvation stress the profile remained sensitive as with the non-stressed control. Acid-adapted RECC 3 exposed to secondary stress changed the sensitive profile to resistant for erythromycin whereas exposure to the secondary stress of acid as well as acid + aerobic changed the profile to intermediate with chloramphenicol.

Table 4. Antibiotic profiles of *C. jejuni* poultry isolate POCC 13 (S: sensitive, I: intermediate or R: resistant) after acid-adaptation and exposure to secondary stresses. The profiles were compared with the control group with no stress exposure.

Antibiotics used	Conc. of antibiotics (in µg)	Non-stressed	Acid-adapted *C. jejuni* POCC 13 exposed to different secondary stresses		
			Starvation + Microaerobic	Acid + Microaerobic	Acid + aerobic
Ampicillin	10 µg	S	S	S	S
Chloramphenicol	30 µg	S	S	S	S
Clindamycin	2 µg	S	S	S	S
Ciprofloxacin	5 µg	S	**I**	S	**R**
Erythromycin	15 µg	S	S	S	S
Gentamicin	10 µg	S	S	S	**R**
Kanamycin	30 µg	R	**S**	**S**	**S**
Nalidixic acid	30 µg	R	R	**S**	R
Streptomycin	10 µg	I	**S**	**S**	**R**
Tetracycline	30 µg	R	R	R	R
Vancomycin	30 µg	R	R	R	R

Table 5. Antibiotic profiles of *C. jejuni* poultry isolate RECC 3 (S: sensitive, I: intermediate or R: resistant) after acid-adaptation and exposure to secondary stresses. The profiles were compared with the control group with no stress exposure.

Antibiotics used	Conc. of antibiotics (in µg)	Non-stressed	Acid-adapted *C. jejuni* RECC 3 exposed to different secondary stresses		
			Starvation + Microaerobic	Acid + Microaerobic	Acid + aerobic
Ampicillin	10 µg	R	R	R	S
Chloramphenicol	30 µg	S	S	**I**	**I**
Clindamycin	2 µg	S	S	**R**	**R**
Ciprofloxacin	5 µg	R	R	R	**I**
Erythromycin	15 µg	S	S	**R**	S
Gentamicin	10 µg	S	S	**R**	**R**
Kanamycin	30 µg	S	S	S	S
Nalidixic acid	30 µg	R	R	R	**S**
Streptomycin	10 µg	S	S	S	S
Tetracycline	30 µg	R	R	R	R
Vancomycin	30 µg	R	R	R	R

4. Discussion

Foodborne bacteria encounter a variety of stresses in the processing environments, in foods and inside their hosts. These stresses have been found to induce adaptive responses in these bacteria along with changes in the cell which affects their innate antimicrobial susceptibility [15]. Sublethal food preservation stresses such as salt (>4.5%), reduced pH (<5.0) and high temperature (45˚C) were found to produce significantly different antibiotic sensitivity patterns for foodborne pathogens such as *E. coli*, *Salmonella* Typhimurium and *Staphylococcus*. Some of these stresses were found to induce permanent changes in antibiotic sensitivity profiles even after removal of the stresses [16]. However, research on the effects of stress-adaptation and subsequent exposure to secondary stresses on the antibiotic sensitivity profiles of foodborne bacteria is limited.

Various types of secondary stresses produce different types of responses in the bacteria including changes in their antimicrobial susceptibilities. All the four *C. jejuni* isolates showed differences in sensitivity profiles against all the antibiotics used in the present study after stress-adaptation and exposure to secondary stresses except for vancomycin. These differences in the sensitivity profile were found to be dependent on the isolate, acid-adaptation, and subsequent exposure to secondary stresses.

Acid-adapted *C. jejuni* human isolate (81 - 176) was

found to be resistant to nalidixic acid when it was exposed to the secondary stress of starvation. Starvation is known to activate a stringent response which produces a transcriptional switching resulting in reduced expression of genes affecting growth and increased expression of genes required for the survival of the bacteria [20]. Stringent response is also known to produce increased levels of alarmone guanosine 5'-(tri)diphosphate 3'-diphosphate [(p)ppGpp] which is known to affect the cell physiology in many ways including antimicrobial susceptibility [21]. Increase in ppGpp under starvation conditions has shown to increase the resistance of *E. coli* toward antibiotics like penicillin, trimethoprim, gentamicin and polymixin B [22]. The stringent response also decreases the production of pro-oxidant molecules such as 4-hydroxy-2-alkylquinolines (HAQs) and increases the antioxidant defenses which in turn increases oxidative killing by bactericidal antibiotics such as aminoglycosides, B-lactam, cationic and fluoroquinolone groups of antibiotics [23]. In our study, however, when the acid-adapted *C. jejuni* poultry isolate, PRCC 3, was exposed to starvation stress it was found to be more sensitive to nalidixic acid. Similarly acid-adapted *C. jejuni* poultry isolate, POCC 13, was found to be more sensitive to kanamycin when exposed to starvation. It might be assumed that some changes may be happening in these isolates during acid-adaptation which might be changing the resistance pattern when they are subsequently exposed to starvation. The *C. jejuni* poultry isolate, RECC 3, however did not show any difference in the sensitivity to the various antibiotics after exposure to the secondary stress of starvation compared to its control.

Exposure to oxygen or an oxidative stress was found to induce the genes of the multidrug efflux system, MaxXY-OprM of *Pseudomonas aeruginosa* that are known to be activated upon exposure to various antimicrobials [24]. *C. jejuni* also possess several drug efflux pumps of different families, but many of them are yet to be functionally characterized. The main drug efflux pump of *C. jejuni* CMeABC is an RND (Resistance Nodulation Division) type of efflux transporter which is known to confer resistance to several antibiotics [25]. This drug efflux pump was also found to be involved in bile resistance as well as colonization of *C. jejuni* in the chicken gut [26]. In this research, the poultry *C. jejuni* isolates were found to be resistant to ampicillin, gentamicin, ciprofloxacin and clindamycin when compared to their non-stressed controls. Hence, it might be assumed that similar to *P. aeruginosa*, the efflux systems of *C. jejuni* may be playing an important role in the antibiotic resistance when these bacteria are exposed to a secondary stress of acid and exposure to oxygen.

The exposure of acid-adapted *C. jejuni* poultry isolates to a sublethal pH of 4.5 was found to increase their sensitivity to antibiotics such as nalidixic acid, kanamycin and ampicillin. However, under this condition the retail isolate RECC 3 was found to be more resistant to erythromycin. These changes might be due to sudden mutations in the genome as stress-induced mutagenesis is found to increase when bacteria are exposed to stresses like starvation, exposure to oxygen, low pH, temperature extremes and exposure to antibiotics [27].

5. Conclusion

The results of this research indicate that stresses commonly encountered in the food and poultry processing environments when given in a sequential pattern may give rise to more antibiotic sensitive populations of *C. jejuni*. However, some stresses are found to produce a reverse effect by increasing the resistance of *C. jejuni*, which may help them to survive further stresses such as passage through the gastrointestinal tract. This type of a stress-adaptation could play a major role in the response of the bacteria towards antibiotics. Additional research is needed to determine the types of stresses that could increase the susceptibility of *C. jejuni* towards antibiotics. Further studies could also be conducted to determine the mechanisms involved in the response of *C. jejuni* towards antibiotics under the influence of multiple stresses.

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