

Research article

Bile-Salt Stress Activation of *cadF* and *flaA* Virulence Gene Expression in Zoonotic *Campylobacter jejuni* from Poultry with HPLC-Based Organic Acid Profiling in Al-Diwaniyah City, Iraq

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ABSTRACT

Campylobacter jejuni is a major zoonotic foodborne pathogen associated with poultry and poultry-derived products. This study evaluated whether bile-salt stress alters the expression of *cadF* and *flaA* in poultry-origin *C. jejuni* isolates and whether these changes correlate with HPLC-detected extracellular organic acid remodeling. A total of 160 poultry samples were tested for *Campylobacter* using selective culture, biochemical, and molecular methods. Twenty-two isolates of *C. jejuni* positive for *cadF* and *flaA* were exposed to 42 °C and bile-salt stress (0.15%, 6 h). Gene expression was quantified by RT-qPCR using *rrs* as the reference gene, while organic acids were measured by HPLC. *Campylobacter* spp. were recovered from 38/160 samples, giving an overall isolation rate of 23.8%. *C. jejuni* represented 26/38 isolates, or 68.4%. Among *C. jejuni* isolates, 22/26 (84.6%) were positive for both *cadF* and *flaA*. Bile-salt stress significantly increased *cadF* expression by 2.36-fold and *flaA* expression by 2.91-fold compared with the control condition. HPLC showed increased acetate from 34.5 ± 11.8 to 64.8 ± 18.6 mg/L, succinate from 8.9 ± 3.7 to 19.6 ± 7.4 mg/L, and format from 12.7 ± 5.1 to 24.3 ± 8.9 mg/L, while lactate decreased from 55.2 ± 17.4 to 31.7 ± 12.8 mg/L. *flaA* expression correlated positively with format accumulation, while *cadF* expression correlated positively with acetate concentration. Bile salts did not act only as an inhibitory intestinal barrier for poultry-origin *C. jejuni*; instead, bile exposure promoted coordinated virulence and metabolic adaptation that may support intestinal colonization and zoonotic transmission.

Keywords: *Campylobacter jejuni*; bile salts; foodborne pathogens; organic acids; poultry; RT-qPCR; virulence gene expression; zoonotic bacteria.

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1. INTRODUCTION

Campylobacter jejuni is a zoonotic bacterium of primary public health concern as a food-borne pathogen. It is one of the most frequent causes of bacterial gastroenteritis globally, with poultry being identified as one of the major reservoirs of *C. jejuni* for humans [1]. Infected humans are thought to acquire the bacterium from the consumption of undercooked contamin-

ated poultry, cross-contaminated ready-to-eat foods, raw milk, and other contaminated sources of water [2]. *C. jejuni* is able to colonize the intestinal tract of chickens and other birds without causing obvious pathology, and therefore, poultry is an important subclinical reservoir in the food production system [3]. As a microaerophilic and thermophilic bacterium, *C. jejuni* adapted

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to the avian intestinal system can survive and thrive in the intestinal tract of birds at their normal body temperature, interact with intestinal mucus, and exhibit motility and chemotaxis as well as survive various types of stress [4].

The major pathogenicity of the *C. jejuni* relies on a number of genes such as motility, adhesion, invasion, and toxins as well as the genes that mediate stress and immunity [5]. The *flaA* gene codes for the major flagellin protein and is critical for motility and penetration of intestinal mucus, colonization and interaction with the host [6]. The *cadF* gene codes for a fn-binding outer membrane protein, which is involved in adhesion to the epithelial cells of the host, and is regarded as a major adhesion determinant in the pathogenicity of *Campylobacter* [7]. Therefore, this model of stress model is biologically relevant of understanding *C. jejuni* colonization in the intestinal tract. Bile salts are of particular interest, as they are experienced by the bacterium en route to the intestinal tract. They may also provide antimicrobial stress and act as environmental signals [8].

Bile adaptation in bacteria encompasses more than the ability to survive exposure. Mechanisms such as expression of virulence genes, motility, membrane adaptation, efflux, and metabolism may also be influenced by bile [9]. Some metabolic changes may be monitored using organic acids and short-chain metabolites such as lactate, acetate, succinate, and formate. These organic acids and metabolites may reflect a change in substrate, energy metabolism, and redox [10].

The relationship between bile-responsive virulence gene expression and changes in extracellular organic acids in poultry-derived *C. jejuni* remains poorly characterized, particularly in Iraq [8,9].

The current study aimed to determine the presence of *C. jejuni* in poultry samples from Al-Diwaniyah City, Iraq. This study also evaluates how bile-salt stress affects *cadF* and *flaA* expression. It also examined whether these changes are associated with HPLC-measured shifts in extracellular organic acids.

2. MATERIALS AND METHODS

2.1. Study Design

A cross-sectional laboratory study was conducted to evaluate poultry-associated *Campylobacter jejuni* and to determine whether bile-salt exposure was associated with changes in *cadF* and *flaA* transcription and extracellular organic-acid concentrations. The workflow comprised sample collection, selective enrichment and isolation, phenotypic screening, single-colony purification, species-specific molecular confirmation, virulence-gene screening, bile-salt exposure, RT-qPCR, HPLC, and isolate-level statistical analysis. The same confirmed *C. jejuni* isolate served as its own control in the bile-stress experiment.

2.2. Sample Collection

A total of 160 poultry-related samples were obtained in Al-Diwaniyah City, Iraq: 50 fresh chicken carcass swabs, 40 frozen chicken carcass swabs, 40 liver/gizzard samples, and 30 cloacal swabs from apparently healthy broiler chickens. Carcass swabs were taken from the breast, thigh, and wing surfaces. Liver and gizzard specimens were transferred individually into sterile polyethylene sampling bags, and cloacal samples were collected with sterile transport swabs. Samples were transported at 4–8°C in an insulated container and

processed within 4 h. Each specimen was assigned a unique code recording sample type, source, collection date, and transport temperature.

2.3. Preparation of Poultry Samples

For liver and gizzard specimens, 25 gm was aseptically transferred to a sterile stomacher bag containing 225 mL Bolton Broth Base (Oxoid, Thermo Fisher Scientific, United Kingdom; Cat. No. CM0983) supplemented with Bolton Broth Selective Supplement (Oxoid; Cat. No. SR0183E) and 5% lysed horse blood. Each carcass or cloacal swab was immersed in 10 mL of the same supplemented Bolton broth and vortexed for 60 s. Enrichment cultures were incubated for 4 h at 37°C followed by 44 h at 42°C under microaerophilic conditions.

2.4. Microaerophilic Enrichment Conditions

Microaerophilic incubation was generated with CampyGen gas-generating sachets (Oxoid, Thermo Fisher Scientific, United Kingdom; Cat. No. CN0025A) in sealed jars, providing approximately 5% O₂, 10% CO₂, and 85% N₂. An atmosphere indicator was included in every jar, and all enrichment and culture incubations were performed at 42°C unless otherwise specified.

2.5. Isolation and Purification of *Campylobacter* spp.

After enrichment, a loopful of broth was streaked onto modified charcoal cefoperazone deoxycholate agar (mCCDA) prepared from Modified *Campylobacter* Blood-Free Selective Agar Base (Oxoid; Cat. No. CM0739B) and CCDA Selective Supplement (Oxoid; Cat. No. SR0155E). Plates were incubated for 48 h at 42°C under microaerophilic conditions. Small, moist, greyish, spreading, or dew-drop-like colonies were selected. Candidate colonies were transferred to Columbia Agar Base (Oxoid; Cat. No. CM0331) containing 5% defibrinated sheep blood and subcultured to obtain discrete pure colonies.

2.6. Initial Phenotypic Characterization

Purified colonies were screened by Gram staining, wet-mount motility, oxidase testing, catalase testing with 3% H₂O₂, growth at 42°C under microaerophilic conditions, and hippurate hydrolysis. Curved or spiral Gram-negative rods showing characteristic darting motility and positive oxidase and catalase reactions were retained for molecular confirmation. Hippurate hydrolysis was used as a supportive phenotypic discriminator for *C. jejuni* and was not used as the sole species-identification criterion.

2.7. Microorganism, Species Confirmation, and Resolution of Mixed Samples

The microorganism evaluated in the bile-stress sub-study was pure-culture *C. jejuni*. To resolve samples containing more than one thermophilic *Campylobacter* species, three to five spatially separated colonies from each positive primary culture were picked independently and subcultured twice on Columbia blood agar. DNA from each purified colony was tested separately by species-specific real-time PCR targeting *mapA* for *C. jejuni* and *ceuE* for *C. coli*. A sample was classified as *C. jejuni* only when purified colonies showed the *mapA*-positive/*ceuE*-negative profile, as *C. coli* only when purified colonies showed the *ceuE*-positive/*mapA*-negative profile, and as mixed *C. jejuni/C. coli* only when separately purified

colonies from the same original sample yielded the two different species-specific profiles. Thus, “mixed” referred to the original sample rather than a single bacterial isolate. Mixed samples were excluded from the pure-isolate bile-stress expression study.

2.8. Genomic DNA Extraction and Quality Control

Genomic DNA was extracted from 48-h purified cultures with the QIAamp DNA Mini Kit (QIAGEN, Germany; Cat. No. 51304). Bacterial biomass was suspended in 180 µL ATL buffer, mixed with 20 µL proteinase K, and incubated at 56°C for 30 min. After addition of 200 µL AL buffer, samples were vortexed and incubated at 70°C for 10 min. Absolute ethanol (200 µL) was added, the lysate was transferred to the spin column, and the column was centrifuged at 8000 × g for 1 min. Columns were washed with 500 µL AW1 and 500 µL AW2, and DNA was eluted in 100 µL AE buffer. DNA concentration and A260/A280 purity were measured with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA); DNA with an A260/A280 ratio of 1.7–2.0 was used for molecular assays and stored at –20°C.

2.9. Species-Specific Real-Time PCR

Species differentiation used short-amplicon primers published by Price et al. [11]. The *C. jejuni mapA* assay generated a 67-bp product using 5'-GCTAGAGGAATAGTTGTGCTTGACAA-3' and 5'-TTACTCACATAAGGTGAATTTGATCG-3'; the *C. coli ceuE* assay generated a 72-bp product using 5'-CAAGTACTGCAATAAACTAGCACTACG-3' and 5'-AGCTATCACCCCTCATCACTCATACTAATAG-3'. Each 20-µL SYBR Green reaction contained 10 µL 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA; Cat. No. K0221), 0.8 µL of each 10-µM primer, 2 µL DNA template, and 6.4 µL nuclease-free water. Amplification was performed on an Applied Biosystems StepOnePlus Real-Time PCR System with 50°C for 2 min and 95°C for 10 min, followed by 30 cycles of 95°C for 15 s and 59°C for 30 s. Dissociation analysis was performed from 61°C to 95°C. Each purified colony was tested in duplicate together with no-template controls and species-control DNA; discordant duplicate reactions were repeated from a newly purified colony.

Table 1. Species-specific short-amplicon primers used to distinguish *C. jejuni* and *C. coli*.

Species / target	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon (bp)	Reference
<i>C. jejuni</i> / <i>mapA</i>	GCTAGAGGAA TAGTTGTGCT TGACAA	TTACTCACATAAG GTGAATTTGATC G	67	[11]
<i>C. coli</i> / <i>ceuE</i>	CAAGTACTGC AATAAACT AGCACTACG	AGCTATCACCCCTC ATCACTCATACTA ATAG	72	[11]

2.10. Storage and Eligibility of *C. jejuni* Isolates

Pure molecularly confirmed *C. jejuni* isolates were stored in Mueller–Hinton broth (HiMedia Laboratories, India; Cat. No. M391) containing 20% glycerol (Merck, Germany; Cat. No. 104094) at –80°C. Before the stress experiment, isolates were revived on Columbia blood agar for 48 h at 42°C under microaerophilic conditions and screened for *cadF* and *flaA*. All 22 isolates positive for both genes were included; no eligible isolate was subsampled or excluded from the expression/HPLC analysis.

2.11. Virulence-Gene Screening and RT-qPCR Primers

The *cadF* and *flaA* primer pairs were taken from the published RT-qPCR study of Chaisowong et al. [12] and generate 94-bp and 113-bp amplicons, respectively. The *rrs* reference-gene primers were those used in the published *C. jejuni* RT-qPCR study of Taha-Abdelaziz et al. [13], which evaluated reference-gene stability and selected *rrs* for normalization. Lyophilized primers were supplied by Macrogen (South Korea) or Metabion (Germany), reconstituted to 100 µM stocks, and diluted to 10 µM working solutions. The same short-amplicon *cadF* and *flaA* pairs were used for genomic screening and transcript quantification.

Table 2. Short-amplicon RT-qPCR primers used for virulence-gene expression analysis.

Gene	Function	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon (bp)	Ref
<i>cadF</i>	Fibronectin-binding adhesin	TTCTATGG TTTAGCAG GTGGAG	TTACACCCGC GCCATAAT	94	[12]
<i>flaA</i>	Major flagellin / motility	GGATGGC GATAGCAG ATAGTTT	CTCATCCATA GCCTTATCAG CA	113	[12]
<i>rrs</i>	16S rRNA reference gene	AAGGGCCA TGATGACT TGAC	AGCGCAACC CACGTATTTA G	107	[12]

2.12. Preparation of Bacterial Inoculum for Bile-Salt Stress

Each *cadF/flaA*-positive *C. jejuni* isolate was revived on Columbia blood agar containing 5% defibrinated sheep blood for 48 h at 42°C under microaerophilic conditions. A fresh 18-h culture was suspended in Mueller–Hinton broth and adjusted to 0.5 McFarland (approximately 1.5×10^8 CFU/mL). A 1:100 dilution was prepared in fresh Mueller–Hinton broth immediately before inoculation of the experimental cultures.

2.13. Bile-Salt Stress Treatment and Replication

Bile Salts No. 3 (Oxoid, Thermo Fisher Scientific, United Kingdom; Cat. No. LP0056B) was prepared as a sterile 10% (w/v) stock in phosphate-buffered saline and passed through a 0.22-µm membrane. For each of the 22 isolates, two matched conditions were established: Mueller–Hinton broth without bile salts (control) and Mueller–Hinton broth containing 0.15% (w/v) bile salts. Each tube contained 10 mL final culture volume inoculated with 100 µL standardized bacterial suspension and was incubated for 6 h at 42°C under the same microaerophilic atmosphere. Three parallel culture replicates were prepared for each isolate-condition. These within-isolate replicates were averaged before inferential statistics, so the biological unit of analysis remained the isolate (n = 22). After exposure, cultures were centrifuged at 10,000 × g for 10 min at 4°C; pellets were processed immediately for RNA stabilization/extraction, and supernatants were stored at –20°C for HPLC.

2.14. Total RNA Extraction and DNase Treatment

RNAprotect Bacteria Reagent (QIAGEN, Germany; Cat. No. 76506) was added to each bacterial culture at a 2:1

reagent-to-culture ratio before extraction. Total RNA was isolated with the RNeasy Mini Kit (QIAGEN; Cat. No. 74104). Pellets were resuspended in 700 μ L Buffer RLT containing 1% β -mercaptoethanol, vortexed for 1 min, held for 5 min at room temperature, mixed with 700 μ L 70% ethanol, and loaded onto RNeasy columns. Columns were centrifuged at $8000 \times g$ for 30 s and washed according to the manufacturer's protocol. RNA was eluted in 30–50 μ L RNase-free water. Residual DNA was removed with RNase-free DNase I (Thermo Fisher Scientific, USA; Cat. No. EN0521): 1 μ g RNA, 1 μ L DNase I, 1 μ L $10\times$ reaction buffer, and nuclease-free water to 10 μ L were incubated at 37°C for 30 min; DNase was then inactivated with 1 μ L 50 mM EDTA at 65°C for 10 min.

2.15. RNA Quality Assessment and cDNA Synthesis

RNA concentration and purity were measured with a NanoDrop One spectrophotometer. Samples with A260/A280 values of 1.8–2.1 were used. RNA was maintained on ice during processing and stored at -80°C when not used immediately. Complementary DNA was synthesized from 1 μ g RNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA; Cat. No. K1622). Each 20- μ L reverse-transcription reaction contained 1 μ g RNA, 1 μ L random hexamer primer, 4 μ L $5\times$ reaction buffer, 1 μ L RiboLock RNase inhibitor, 2 μ L 10 mM dNTP mix, 1 μ L RevertAid reverse transcriptase, and nuclease-free water to volume. Reactions were incubated at 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min. cDNA was diluted 1:5 in nuclease-free water and stored at -20°C until RT-qPCR.

2.16. RT-qPCR Gene Expression Assay

RT-qPCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR System using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA; Cat. No. K0221). Each 20- μ L reaction contained 10 μ L $2\times$ master mix, 0.8 μ L 10- μ M forward primer, 0.8 μ L 10- μ M reverse primer (0.4 μ M final concentration each), 2 μ L 1:5-diluted cDNA, and 6.4 μ L nuclease-free water. Every cDNA sample was tested in three technical wells. No-template and no-reverse-transcription controls were included on each plate. The thermal program was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. A melting curve from 65°C to 95°C in 0.3°C increments was used to verify a single amplification product. Technical Ct values were averaged before isolate-level calculations.

2.17. Calculation of Relative Gene Expression

Relative transcription was calculated by the $2^{-\Delta\Delta\text{Ct}}$ method. For each isolate, $\Delta\text{Ct} = \text{Ct}(\text{target}) - \text{Ct}(\text{rrs})$, and $\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{bile-stress}) - \Delta\text{Ct}(\text{control})$. The matched untreated culture of the same isolate was the calibrator and was assigned a relative-expression value of 1.00. The three technical qPCR wells were first averaged, followed by averaging of the three parallel culture replicates to produce one control and one bile-stress value per isolate. These isolate-level values were used in all inferential analyses.

2.18. Preparation of Supernatants for HPLC

Culture supernatants from control and bile-stress conditions were collected after centrifugation at $10,000 \times g$ for 10 min at 4°C and filtered through 0.22- μ m syringe filters (Merck Millipore, Germany; Cat. No. SLGP033RS). Filtrates

were transferred to 1.5-mL HPLC vials with PTFE/silicone septa and stored at -20°C for no longer than one week. Before analysis, samples were thawed at room temperature, vortexed for 30 s, and centrifuged at $12,000 \times g$ for 5 min.

2.19. HPLC Standards and Calibration

Analytical standards were L-lactic acid (Sigma-Aldrich/Merck, Germany; Cat. No. 46937), acetic acid (Cat. No. 71251), succinic acid (Cat. No. S3674), and formic acid (Cat. No. 33015). Individual 1000-mg/L stocks were prepared in HPLC-grade water. Working standards of 5, 10, 25, 50, 100, and 200 mg/L were used to generate external calibration curves from peak area versus concentration. Calibration was accepted at $R^2 \geq 0.995$.

2.20. HPLC Instrumentation and Operating Conditions

Organic acids were quantified with a Shimadzu LC-2030C HPLC system (Shimadzu Corporation, Japan) equipped with a quaternary pump, autosampler, thermostatted column compartment, and UV detector. Separation was performed on an Aminex HPX-87H ion-exclusion column (300 $\times$ 7.8 mm; Bio-Rad Laboratories, USA; Cat. No. 1250140). The mobile phase was 5 mM sulfuric acid prepared from analytical-grade H_2SO_4 (Merck, Germany; Cat. No. 100731) in HPLC-grade water, filtered through a 0.45- μ m membrane, and sonicated for 15 min. Isocratic elution was performed at 0.6 mL/min, 40°C, with a 20- μ L injection volume, UV detection at 210 nm, and a 25-min run time. Analytes were identified by retention-time agreement with standards and quantified from the external calibration curves. Each prepared culture supernatant was injected twice; duplicate injection values were averaged before the three parallel culture replicates were averaged to a single isolate-condition concentration.

2.21. HPLC Quality Control

A mobile-phase blank was injected before each calibration series. A mixed organic-acid quality-control standard was injected after every ten analytical injections to monitor retention-time and detector-response stability. Repeated standard injections were accepted when the relative standard deviation was $<5\%$. Samples showing peak overlap, abnormal peak shape, or retention-time drift >0.2 min were refiltered and reinjected.

2.22. Statistical Analysis and Post-hoc Sensitivity Analysis

Statistical analyses were performed with SPSS version 26 and GraphPad Prism version 10. Isolation and species-profile data were summarized as n/N, percentages, and Wilson 95% confidence intervals; categorical distributions were assessed with chi-square tests. For the bile-stress sub-study, the isolate was the biological unit of analysis ($n = 22$). Parallel culture replicates, RT-qPCR technical wells, and duplicate HPLC injections were averaged hierarchically before inferential testing. Control and bile-stress values from the same isolate were compared with two-sided paired t-tests. Pearson correlation was used for isolate-level associations between virulence-gene expression and organic-acid concentrations. Five prespecified correlation tests were adjusted by the Benjamini–Hochberg false-discovery-rate procedure, with $q < 0.05$ defining FDR significance. Fold-change means are reported with 95% confidence intervals, and Pearson r values

with Fisher-z 95% confidence intervals. A post-hoc sensitivity analysis was performed in G*Power 3.1 [14] for a two-sided paired t-test with $n = 22$, $\alpha = 0.05$, and 80% power; the minimum detectable standardized paired effect was Cohen's $d^2 = 0.63$. This sensitivity analysis was used rather than deriving achieved power from the observed P values.

3. RESULTS

3.1. Isolation Rate of *Campylobacter* spp. from Poultry Samples

Thirty-eight of 160 poultry-related samples were positive for *Campylobacter* spp. (23.8%). Positivity was highest in fresh chicken carcasses (18/50, 36.0%), followed by liver/gizzard samples (11/40, 27.5%), cloacal swabs (6/30, 20.0%), and frozen chicken carcasses (3/40, 7.5%). The distribution differed significantly among sample types ($\chi^2 = 10.52$, $P = 0.015$; Table 3; Figure 1).

Table 3. Distribution of *Campylobacter* spp. according to poultry sample type.

Sample type	Positive examined /	Positivity (%)	Wilson 95% CI (%)
Fresh chicken carcasses	18/50	36.0 ^a	24.1–49.9
Frozen chicken carcasses	3/40	7.5 ^c	2.6–19.9
Chicken liver and gizzard	11/40	27.5 ^{ab}	16.1–42.8
Cloacal swabs	6/30	20.0 ^b	9.5–37.3
Total	38/160	23.8	17.8–30.9
Chi-square		10.52	
P value		0.015	

Different superscript letters indicate significant differences between sample types at $P < 0.05$. Confidence intervals are Wilson 95% intervals.

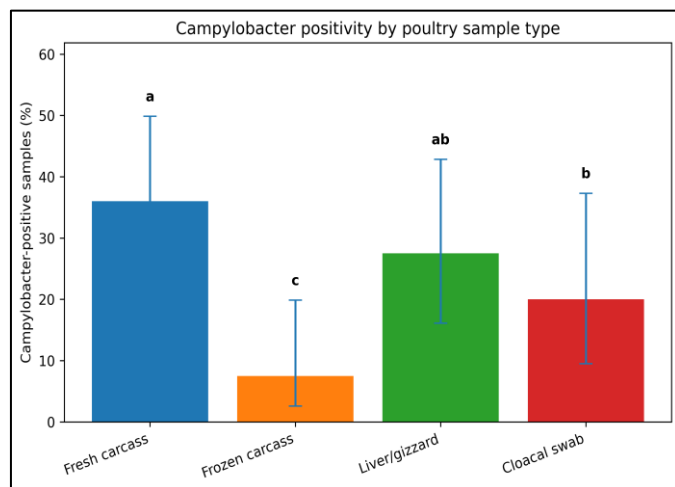


Fig. 1. *Campylobacter* positivity by poultry sample type. Bars show percentages, error bars show Wilson 95% confidence intervals, and superscript letters correspond to the significance groups.

3.2. Species Confirmation and Mixed-Sample Resolution

Among the 38 *Campylobacter*-positive samples, 26 (68.4%) yielded only molecularly confirmed *C. jejuni*, 8 (21.1%) yielded

only *C. coli*, and 4 (10.5%) yielded both species from independently purified colonies. The four mixed profiles therefore represented mixed original samples, not dual-species single colonies. The species-profile distribution was significant ($\chi^2 = 21.68$, $P < 0.001$; Table 4; Figure 2). Mixed samples were not carried into the pure-*C. jejuni* bile-stress experiment.

Table 4. Species profiles among *Campylobacter*-positive samples.

Species profile	No. samples	Percentage	Wilson 95% CI (%)
<i>C. jejuni</i> only	26	68.4 ^a	52.5–80.9
<i>C. coli</i> only	8	21.1 ^b	11.1–36.3
Mixed <i>C. jejuni/C. coli</i>	4	10.5 ^c	4.2–24.1
Total	38	100	
Chi-square		21.68	
P value		<0.001	

Mixed indicates recovery of separately purified colonies of both species from the same original sample. Different superscript letters indicate significant differences at $P < 0.05$.

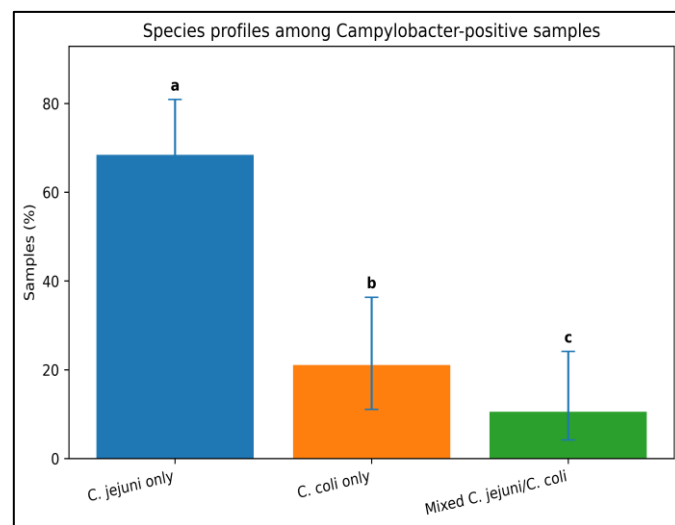


Fig. 2. Species profiles among the 38 *Campylobacter*-positive samples. Error bars show Wilson 95% confidence intervals; different letters correspond to the significance groups.

3.3. Virulence-Gene Profile of Pure *C. jejuni* Isolates

Among 26 pure *C. jejuni* isolates, 22 (84.6%) were positive for both *cadF* and *flaA*, 2 (7.7%) were *cadF*+/*flaA*–, 1 (3.8%) was *cadF*–/*flaA*+, and 1 (3.8%) lacked both targets. The distribution of virulence-gene profiles was significant ($\chi^2 = 48.38$, $P < 0.001$; Table 5; Figure 3). All 22 double-positive isolates were retained for the bile-stress, RT-qPCR, and HPLC sub-study.

Table 5. Virulence-gene profiles of confirmed pure *C. jejuni* isolates.

Gene profile	No. isolates	Percentage	Wilson 95% CI (%)
<i>cadF</i> +/ <i>flaA</i> +	22	84.6 ^a	66.5–93.8
<i>cadF</i> +/ <i>flaA</i> –	2	7.7 ^b	2.1–24.1
<i>cadF</i> –/ <i>flaA</i> +	1	3.8 ^b	0.7–18.9
<i>cadF</i> –/ <i>flaA</i> –	1	3.8 ^b	0.7–18.9
Total	26	100	
Chi-square		48.38	
P value		<0.001	

Different superscript letters indicate significant differences among gene profiles at $P < 0.05$.

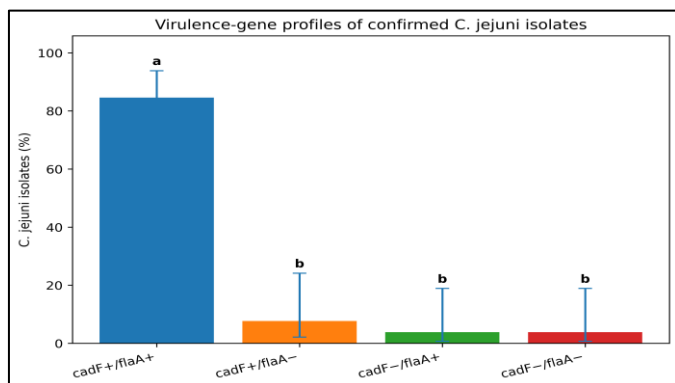


Fig. 3. Virulence-gene profiles of the 26 pure *C. jejuni* isolates. Error bars show Wilson 95% confidence intervals; different letters correspond to the significance groups.

3.4. Effects of Bile-Salt Stress on *cadF* and *flaA* Expression

All 22 eligible double-positive isolates were analyzed as independent biological units. Relative *cadF* expression increased from the matched control calibrator (1.00) to 2.36 ± 0.82 -fold under 0.15% bile salts, with a 95% CI of 2.00–2.72 ($P < 0.001$). Relative *flaA* expression increased to 2.91 ± 1.04 -fold, with a 95% CI of 2.45–3.37 ($P < 0.001$). The magnitude of the observed fold increase was numerically greater for *flaA* than for *cadF* (Table 6; Figure 4).

Table 6. Relative expression of *cadF* and *flaA* after bile-salt exposure.

Gene	Control 42°C	0.15% bile salts, 6 h	Fold-change 95% CI	P value
<i>cadF</i>	1.00 ± 0.00^b	2.36 ± 0.82^a	2.00–2.72	<0.001
<i>flaA</i>	1.00 ± 0.00^b	2.91 ± 1.04^a	2.45–3.37	<0.001

Values are isolate-level mean $\pm$ SD ($n = 22$). Different superscript letters within a row indicate a significant paired difference at $P < 0.05$.

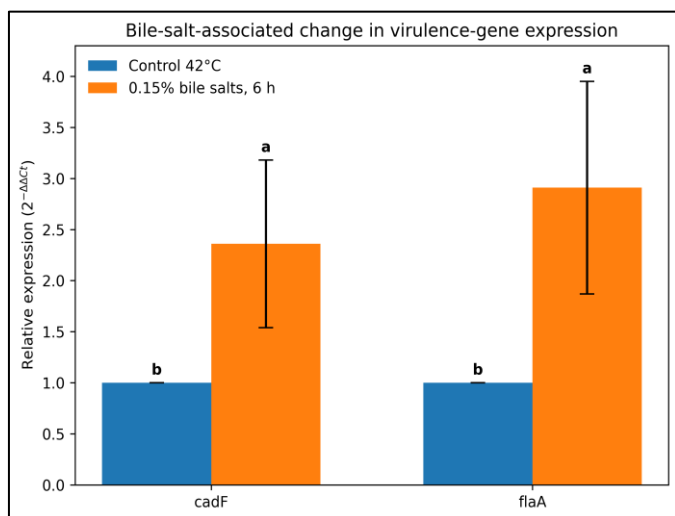


Figure 4. Relative expression of *cadF* and *flaA* in matched control and bile-salt conditions. Bars show isolate-level mean fold expression and error bars show SD ($n = 22$); different letters indicate $P < 0.05$ within each gene.

3.5. HPLC Analysis of Organic Acids under Bile-Salt Stress

Bile-salt exposure was accompanied by marked changes in extracellular organic acids. Acetate increased from 34.5 ± 11.8 to

64.8 ± 18.6 mg/L (+87.8%; $P < 0.001$), succinate from 8.9 ± 3.7 to 19.6 ± 7.4 mg/L (+120.2%; $P < 0.001$), and formate from 12.7 ± 5.1 to 24.3 ± 8.9 mg/L (+91.3%; $P = 0.002$). Lactate decreased from 55.2 ± 17.4 to 31.7 ± 12.8 mg/L (–42.6%; $P < 0.001$). The results therefore showed a bile-associated extracellular shift characterized by increased acetate, succinate, and formate and decreased lactate (Table 7; Figure 5).

Table 7. HPLC-detected organic acids in culture supernatants.

Organic acid	Control 42°C (mg/L)	0.15% bile salts (mg/L)	Change (%)	P value
Lactic acid	55.2 ± 17.4^a	31.7 ± 12.8^b	–42.6	<0.001
Acetic acid	34.5 ± 11.8^b	64.8 ± 18.6^a	+87.8	<0.001
Succinic acid	8.9 ± 3.7^b	19.6 ± 7.4^a	+120.2	<0.001
Formic acid	12.7 ± 5.1^b	24.3 ± 8.9^a	+91.3	0.002

Values are isolate-level mean $\pm$ SD ($n = 22$). Different superscript letters within each row indicate $P < 0.05$ for the matched control-versus-bile comparison.

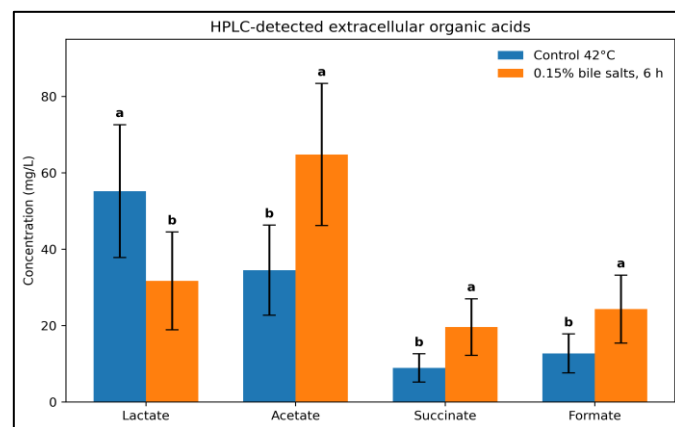


Figure 5. HPLC-detected extracellular organic acids in control and bile-salt conditions. Bars show isolate-level means and error bars show SD ($n = 22$); different letters indicate $P < 0.05$ within each organic acid.

3.6. Correlation Between Virulence-Gene Expression and Organic Acids

After Benjamini–Hochberg correction across the five prespecified correlations, *cadF* expression remained positively associated with acetate ($r = 0.58$, 95% CI 0.21–0.80; $P = 0.005$; $q = 0.013$), and *flaA* expression remained positively associated with formate ($r = 0.63$, 95% CI 0.28–0.83; $P = 0.002$; $q = 0.010$) and negatively associated with lactate ($r = -0.51$, 95% CI –0.77 to –0.11; $P = 0.015$; $q = 0.025$). The *cadF*–succinate association did not remain significant after FDR correction ($r = 0.44$; $P = 0.041$; $q = 0.051$), and the *flaA*–acetate association was not significant ($r = 0.39$; $P = 0.071$; $q = 0.071$; Table 8; Figure 6).

Table 8. Correlation between virulence-gene expression and HPLC organic acids under bile-salt stress.

Correlation	r (95% CI)	P value	FDR q	FDR class
<i>cadF</i> vs acetate	0.58 (0.21–0.80)	0.005	0.013	a
<i>cadF</i> vs succinate	0.44 (0.02–0.73)	0.041	0.051	b
<i>flaA</i> vs formate	0.63 (0.28–0.83)	0.002	0.010	a
<i>flaA</i> vs lactate	–0.51 (–0.77 to –0.11)	0.015	0.025	a
<i>flaA</i> vs acetate	0.39 (–0.04–0.70)	0.071	0.071	b

Pearson correlations use isolate-level values ($n = 22$). Benjamini–Hochberg correction was applied across the five tests. Class a = $q < 0.05$; class b = $q \geq 0.05$.

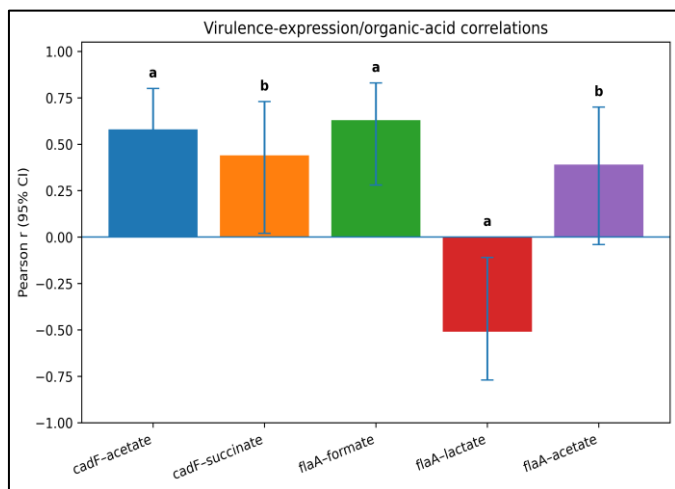


Fig. 6. Pearson correlations between virulence-gene expression and organic-acid concentrations under bile-salt stress. Bars show r and error bars show 95% confidence intervals. Letter a denotes FDR-significant correlations ($q < 0.05$) and b denotes $q \geq 0.05$.

3.7. Post-hoc Sensitivity Analysis

The expression/HPLC sub-study included all 22 eligible *cadF/flaA*-positive *C. jejuni* isolates. For a two-sided paired t-test with $\alpha = 0.05$, $n = 22$ provided 80% power to detect a standardized within-isolate effect of Cohen's $d^z = 0.63$. At 90% power, the corresponding detectable effect was $d^z = 0.73$. Thus, the design had adequate sensitivity for moderate-to-large paired effects while retaining all available eligible biological isolates (Table 9).

Table 9. Post-hoc sensitivity of the paired bile-stress sub-study.

Parameter	Value
Biological unit	Confirmed pure <i>C. jejuni</i> isolate
Number of paired isolates	22
Test	Two-sided paired t-test
α	0.05
Power target	80%
Minimum detectable effect	Cohen's $d^z = 0.63$
Sensitivity at 90% power	Cohen's $d^z = 0.73$

4. DISCUSSION

The present study isolated *Campylobacter* from 23.8% of poultry-related samples. This suggests that poultry products and poultry cloaca may be important sources of zoonotic infection. The highest percentage of *Campylobacter* positivity (36.0%) was found in fresh chicken carcasses, followed by chicken livers and gizzards at 27.5%. Positive samples at different stages of slaughtering, evisceration, and carcass handling, as well as cross-contaminated samples, reinforce the spread of *Campylobacter* among poultry. *Campylobacter* in poultry meat and the carriage of *Campylobacter* by poultry provide support to the hypothesis that meat is a major route of *Campylobacter* infection to humans [1]. Frequent isolation of *Campylobacter* from broiler meat and poultry intestinal samples is in agreement with the present study [15]. However, 23.8% positivity is lower compared to studies that reported high levels of contamination in fresh broiler carcasses. This is likely due to variation in the type of samples taken, seasonal differences, and differences in the transport and culture of samples, as well as whether rinsing of broiler carcasses was

done, or deep tissue samples were taken [14]. It is well documented that freezing reduces the viability of *Campylobacter* and low levels of the organism may be recovered from frozen samples [16].

C. jejuni being the most predominant confirmed isolate is because it is commonly seen as the most prevalent thermophilic *Campylobacter* species found both in poultry and in cases of human campylobacteriosis [2]. *C. jejuni* comprised 68.4 percent of confirmed *Campylobacter* isolates in this study, while *C. coli* made up 21.1 percent of the isolates. These findings reflect the trend that *C. jejuni* is almost always found in greater amounts than *C. coli* in both poultry and human cases of infections [17]. However, having *C. coli* in the isolates is of great concern from an epidemiological standpoint due to this species also being zoonotic and possessing the potential to carry antimicrobial resistance and genes associated with virulence and pathogenicity [18]. This study is in contrast to others, which have found higher occurrences of *C. coli* in certain poultry products, from other mixed production systems, and from the other cases studied. These differences are likely attributable to differences in geography, management of the farm, and the dynamics of the intestinal colonization and contamination at the slaughterhouses [19].

The *cadF/flaA*-positive profile was found in 84.6% of confirmed *C. jejuni* isolates, indicating that the majority of isolates possessed both adhesion and motility factors. These findings are consistent with those of other researchers, who also reported the co-occurrence of *cadF* and *flaA* in *C. jejuni* isolates from poultry. *C. jejuni* isolates that possess both *cadF* and *flaA* are likely to be more pathogenic as *cadF* facilitates *C. jejuni* adhesion to and invasion of intestinal epithelial cells and *flaA* provides *C. jejuni* with the means of actively traversing the intestinal lumen. As mentioned previously, the *flaA* gene has also been reported to be present in *C. jejuni* isolates from broiler chickens [19]. In general, the presence of *flaA* and *cadF* in *C. jejuni* isolates from poultry has been well documented in the literature. In most of the studies that reported low frequencies of *flaA* and *cadF* in chicken or human isolates, the loss of virulence factors was attributed to reduced sub-culturing, variability of the isolates, and selection of the genes to be amplified based on the primers used [13,20].

Bile-salt stress resulted in stronger expression of *cadF* and *flaA*. Expression of *flaA* and *cadF* increased to 2.91 and 2.36 times the control, respectively. These results suggest that exposure to bile may serve as an intestinal signal that primes *C. jejuni* for host transit by coordinating motility and adhesion. The rationale for the greater response in *flaA* is that motility is needed to penetrate intestinal mucus and move to preferred locations within the gut [5]. The response in *cadF* expression suggests that, in addition to adhesion and motility, bile stress may prime *C. jejuni* for adhesion to intestinal epithelial cells. This aligns with bile stress and related studies that suggest *C. jejuni* and bile-related stress may influence *C. jejuni*'s viability, expressed virulence, and interaction with the host [8]. This is also agreeable with the studies that suggest bile salts influence the membranes of *C. jejuni* and adapt the bacteria to membrane efflux and active stress response [9]. Even though there is diversity in the responses of *C. jejuni*, stress responses and the responses to bile salts vary depending on the strain, the exposure, and the environment [4]. Bile-responsive membrane adaptation and CmeABC-mediated efflux provide plausible mechanisms supporting bile tolerance and intestinal colonization [9,20].

HPLC results suggested bile-salt stress leads to increases in acetate, succinate, and formate levels, but decreases lactate

levels. The results showed that bile-salt exposure impacted the use of metabolic substrates and intermediary metabolism. The decrease in lactate may be due to the increased lactate consumption [5]. This may be relevant because lactate can support the growth of *C. jejuni* during intestinal infection. Increased levels of acetate may be due to the altered flux of Acetyl-CoA and/or the impact of bile on the metabolism of energy for the adaptive response to stress. Increased levels of succinate and formate may be due to the impact of bile on stress-related microaerophilic conditions and the modulation of pathways associated with respiration and redox balance. *C. jejuni*'s metabolism is impacted greatly by the available host metabolites, oxygen levels, and intestinal chemical stresses. These results also support the use of HPLC as a simple stress-related extracellular metabolic shift technique over the need for approaches in bacterial metabolomics [1].

The strong positive correlation between *cadF* expression and acetate concentration may indicate that adhesion and related activation may be linked to the remodeling of metabolism related to acetate. There is a positive correlation between the expression of *flaA* and the concentration of formate. This may indicate that activation of motility is associated with changes in redox-related metabolism [13]. The negative correlation between *flaA* and lactate may indicate that isolates with a greater response of motility also had a greater consumption of lactate under stress caused by bile. This is significant because it connects a functional colonization gene to a measurable metabolic signal detected by HPLC. Connections of metabolic state and the regulation of virulence have been found in the most recent studies on the pathogenesis of *C. jejuni*, where the acquisition of nutrients, as well as motility and adaptation to the host, are all linked. The current study illustrates, in a concise manner, that bile stress may not only represent a challenging condition in the intestinal tract but also facilitate a coordinated response of virulence and metabolism [9].

From a public-health perspective, the results indicate that poultry-derived *C. jejuni* isolates should be considered as actively carrying virulence-associated genes that respond to bile intestinal exposure by upregulating motility and adhesion genes. This is significant because poultry infection prior to cooking or cross-contamination during food preparation may expose consumers to strains already able to rapidly adjust to intestinal environments [1]. These findings support strict slaughter hygiene, prevention of raw-poultry cross-contamination, cold-chain control, and thorough cooking.

Limitations. The stress sub-study included 22 isolates, one bile concentration and exposure time, an in-vitro design, and a single-city sample; *rrs* stability was not independently validated.

5. CONCLUSION

This study showed that poultry-related samples from the Al-Diwaniyah region potentially contain zoonotic *Campylobacter* spp. with *C. jejuni* as the likely dominant species. Most *C. jejuni* isolates possessed *cadF* and *flaA*, revealing an adhesion–motility virulence profile. Bile-salt stress at a concentration of 0.15% induced the expression of *cadF* and *flaA*, with a greater effect on *flaA*. HPLC analysis revealed that bile stress induced a significant metabolic shift with the accumulation of acetate, succinate, and formate and depletion of lactate. The observed relationships between the concentration of organic acids and the expression of virulence genes suggest that exposure to bile may signal *C. jejuni* strains from poultry to elicit pathogenic and metabolic adaptations.

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Conflict of interest

The authors declare no conflicts of interest.

Ethical Approval

The Scientific Research Ethics Committee of the College of Veterinary Medicine at the University of Al-Qadisiyah reviewed the study protocol. The authors submitted a scanned copy of the Dean of the College of Veterinary Medicine, University of Al-Qadisiyah's signed official letter (No. 1176, dated 17 March 2026) supporting the ethical approval statement, and a copy of this letter is retained in the institutional research archive. The Committee determined that formal experimental animal ethics approval was not required because the study involved the collection and laboratory examination of samples obtained from poultry for the isolation and characterization of *Campylobacter jejuni*. No experimental infection, treatment, manipulation, or other invasive procedures were performed on the animals specifically for this research. Permission to collect samples was obtained from the relevant farm owners prior to sampling. All sampling and laboratory procedures were conducted in accordance with the institutional guidelines for animal welfare and applicable ethical principles.

Author contributions

Hussein Kareem1. Conceptualization; Investigation; Methodology; Data curation; and Writing – original draft.

Amaar Nabeel Al_Jubouri2: Investigation; Methodology; Formal analysis; Validation; Visualization; and Writing – review and editing.

Mustafa Sabbar Mhr3: Resources; Supervision; Project administration; Validation; and Writing – review and editing.

Yaseen MM. Conceptualization; Methodology; Supervision; Project administration; Writing – original draft; and Writing – review and editing.

All authors reviewed and approved the final manuscript and agreed to be accountable for all aspects of the work

AI Declaration

During the preparation of this work, the authors used ChatGPT (GPT-5.6; OpenAI, 2026) to improve language, readability, and formatting. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Data availability

Data will be made available on request.

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