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Antioxidant activity and influence of *Citrus* byproduct extracts on adherence and invasion of *Campylobacter jejuni* and on the relative expression of *cadF* and *ciaB*

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Abstract Adherence and invasion to cells are the key processes during infection development by *Campylobacter jejuni* (*C. jejuni*). In this study, extracts from the byproducts of *Citrus limon*, *Citrus aurantium*, and *Citrus medica* were added to the cultures of *C. jejuni,* and the adherence and invasion of *C. jejuni* to HeLa cells and the expression of *cadF* and *ciaB* genes were analyzed. The relative expression of the genes was determined by quantitative reverse transcription PCR (qRT-PCR). The antioxidant activity was determined using spectrophotometric methods. Byproduct extracts at sub-inhibitory concentrations affected the adherence (reduced 2.3 to 99%) and invasion (reduced 71.3 to 99.2%) to the HeLa cells. The expression of *cadF* and *ciaB* was reduced from 66 to 99% and from 81 to 99%, respectively. The total phenolic content of the byproducts varied from 92 to 26 mg GAE/g and the total flavonoids varied from 161 to 29.29 mg QE/g. *C. aurantium* showed the highest percentage of radical scavenging activity (RSA, 90.1). These extracts can prove as effective alternatives for devising new strategies to control *Campylobacter* infections.

Keywords: *Campylobacter*, adhesion, invasion, citrus extract, antimicrobial activity, antioxidant activity

Introduction

Campylobacter jejuni is one of the most important foodborne pathogens in the world. Although it primarily causes self-limiting diarrhea, *C. jejuni* is also associated with severe neuropathologic disorders such as Guillain–Barré and Reiter syndromes (1). Upon consuming *C. jejuni*-contaminated water or food items such as poultry or raw milk (2), the pathogen interacts with the host cells. Adherence is a key step in the establishment of the infection (3). In this process, fibronectin-binding protein (CadF), which is expressed in all *C. jejuni* and *C. coli* strains (4), plays an important role by binding to the cell matrix protein, fibronectin (5). Invasion antigen B (CiaB), the first secreted factor identified in *C. jejuni*, has an important role in invasion and has been identified as essential for the secretion of other Cia proteins (4).

Recent observations of the antibiotic resistance for some *Campylobacter* strains indicate a need to develop new approaches to block not only growth but also key steps during the infection process (6). Compounds present in plants have been historically studied for their antioxidant and pharmacological activities. *Citrus* plants are among the world's major fruit crops and can be consumed fresh or as processed products. Specifically, extracts from the *Citrus* species show a wide spectrum of antimicrobial activity (7) and hold potential for use in antioxidant-based therapies against cancer, inflammation, and heart disease (8).

Citrus oils and derivatives are generally recognized as safe by the Food and Drug Administration. The juice industry wastes large amounts of fruit byproducts, especially seeds, bagasse, and peels (50–65% of total fruit weight) (9,10). Use of *Citrus* byproducts is growing and studies suggest their potential use as alternatives to prevent fatty acid deterioration in the food industry (9). These byproducts contain high amounts of phenolic compounds such as phenolic acids and flavonoids (which are responsible for the antioxidant activity, 11) and are present in high concentrations (up to 15%) in *Citrus* peels and seeds (9). Although the content of these compounds can be influenced by many factors and could vary during harvest, postharvest, and among species (12-14), studies about their use as alternatives for effective antimicrobials have increased because of the development of antibiotic resistance by various microorganisms, including the *Campylobacter* species (6).

Pathogenesis of *Campylobacter* is initiated with adherence to intestinal epithelial cells, followed by internalization by bacterial invasion. Once inside the cell, a cytolethal distending toxin is produced that results in host cell death (3). Although *Citrus* extracts

have been tested for their ability to inhibit the growth of *C. jejuni* (15-17), little information is available about their effects on pathogenesis. Therefore, the purpose of this study was to evaluate the influence of *Citrus* byproduct extracts on the adherence and invasion processes of *C. jejuni* to HeLa cells and to assess the relative expression levels of the genes involved in these processes (*cadF* and *ciaB*). In addition, the antioxidant activity of these compounds was determined.

Materials and Methods

***Campylobacter* strains and growth conditions** The strain used in this study, *C. jejuni* NCTC 11168, was obtained from the American Type Culture Collection (Manassas, VA, USA). The *C. jejuni* strain NADC 5653 was isolated from chicken (provided by Dr. Irene Wesley, USDA-ARS-NADC, Ames, IA, USA). *C. jejuni* strains 180ip and 238ip were isolated from patients with diarrhea (provided by Dr. Guillermo Ruiz-Palacios, National Institute of Medical Sciences and Nutrition, Mexico City, Mexico). The *Campylobacter* strains were maintained at −80°C in 20% glycerol brain–heart infusion broth (Difco Laboratories, Sparks, MD, USA) supplemented with 0.6% yeast extract. Fresh cultures were grown in Mueller–Hinton broth (MHB, Difco Laboratories) or Mueller–Hinton agar (Difco Laboratories) supplemented with 5% (v/v) defibrinated horse blood (MHS, 18) under microaerobic conditions (42°C, 10% CO_2) in a CO_2 incubator (Shell Lab, Cornellius, OR, USA).

Extraction of *Citrus* byproducts *Citrus* fruits, *viz. Citrus limon* (lime), *Citrus aurantium* L. (bitter orange), and *Citrus medica* L. (citron), free from any physical defects and at the commercial maturity state, were collected from plantations in Nuevo Leon, México. Fruits were washed and only the peels, seeds, and bagasse were used for the experiments. The raw material was dried in an oven (50–55°C) and then milled. Twenty-five grams of each sample material was macerated with 100 mL of 96% ethanol at 25°C for 48 h. The samples were filtered (Whatman no. 1 paper) and concentrated in a rotary evaporator at 50°C (R3000; Buchi, Flawil, Switzerland). The small volume obtained was evaporated at room temperature (25°C), suspended in 10 mL of sterile distilled water, and filter sterilized using nitrocellulose membranes (0.45 μm, Millipore, Billerica, MA, USA). An aliquot of each extract was used for the dry weight determination. All extracts were stored at 4°C in a dark environment until required for use (usually ≤12 weeks). Antimicrobial activity of each extract was evaluated each month during this period by the well-diffusion test (19).

Effect of sub-inhibitory concentrations of *Citrus* byproduct extracts on *C. jejuni* growth In the previous studies from our laboratory, we obtained a minimum bactericidal concentration (MBC) against *C. jejuni* strains of 130–225 μg/mL for *C. limon*, 275–325 μg/mL for *C. aurantium*, and 150–250 μg/mL for *C. medica* byproduct extracts. MBCs varied depending on the analyzed strain (16). In this study, we determined the effects of the sub-inhibitory concentrations (lower than MBC) of the byproduct extracts on *C. jejuni* growth with the purpose of using these concentrations for adherence and invasion assays.

Tubes containing 5 mL of MHB were inoculated with 50 μL of fresh *C. jejuni* culture (A_{600} 0.5, ~1×10^7 CFU/mL), followed by the addition of different concentrations of extracts (75, 50, and 25% of MBC, Table 1). Screw cap glass tubes were incubated at 42°C for 24 h in a microaerophilic atmosphere (10% CO_2, mod 307; Thermo Fisher Scientific, Marietta, OH, USA). Serial decimal dilutions were made, plated on MHS, and incubated as above. Bacterial counts were reported as CFU/mL. MiliQ water (e-pure, mod D4641; Barnstead, Dubuque, IA, USA) was used as a negative control.

Adherence and invasion assays HeLa cells (provided by Dra. Rocío Ortiz, Medical School of the Universidad Autónoma de Nuevo León) were grown in monolayers with Dulbecco's minimal essential medium (DMEM, Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% L-glutamine 200 mM (Gibco) at 37°C in a 5% CO_2 atmosphere. For assays, the cells were harvested by trypsinization (0.25% trypsin-EDTA, Gibco), seeded in sterile 24-well cell-culture plates (2×10^5 cells per well; Corning, Costar, Cambridge, MA, USA), and incubated (37°C and 5% CO_2) until confluence. The adherence and invasion assays were conducted according to the methods reported by Castillo *et al*. (18) and Ganan *et al*. (20), respectively, with some modifications.

Adherence assay Bacterial cultures of four *C. jejuni* strains were

Table 1. Minimal Bactericidal Concentrations (MBC) and sub-inhibitory concentration corresponding to 75% of the MBC of citric byproducts against four strains of *C. jejuni*

Citrus by-product	*C. jejuni* strain							
	NCTC 11168		NADC 5653		238ip		180ip	
	MBC[2)]	75% MBC	MBC	75% MBC	MBC	75% MBC	MBC	75% MBC
Citrus limon	225±28[1)]	169	225±21	169	130±23	98	210±21	158
Citrus aurantium L.	325±28	244	325±19	244	275±28	206	315±41	236
Citrus medica L.	250±40	188	230±40	173	150±41	113	250±42	188

[1)]±Standard Deviation

[2)]MBC and Sub-inhibitory concentration are presented in μg/mL

Table 2. Primers utilized in this study for qRT-PCR

Gene	Function	Primer sequence	Size (bp)	Reference
cadF	Fibronectin-binding protein (adherence)	F 5'-TTGAAGGTAA TTTAGATATG-3' R 5'-CTAATACCTA AAGTTGAAAC-3'	400	45
ciaB	Invasion antigen B	F 5'-TGCCCGCCTT AGAACTTACA-3' R 5'-TGCGAGATTT TTCGAGAATG-3'	527	45
glyA	Serine hydroxymethyl transferase (housekeeping gene)	F 5'-CGATGGAACG GATAATCACC-3' R 5'-ATACCTGCAT TTCCAAGAGC-3'	93	46

separately preincubated (42°C/24 h under microaerophilic conditions) in MHB supplemented with 75% of MBC (Table 1) for the *Citrus* byproduct extracts. Bacterial cells were centrifuged (6,000×*g* for 10 min), suspended in 5 mL of DMEM with 1% FBS, and adjusted to 10^8 CFU/mL (A_{600} 0.55). Aliquots (100 μL) were added to microtiter plate wells containing confluent HeLa cells. Infected monolayers were incubated (37°C and 5% CO_2) for 3 h to enable the bacteria to attach to the HeLa cells. The monolayers were washed three times with 500 μL of phosphate-buffered saline (PBS) to eliminate the non-attached bacteria. The adhered bacteria were detached by adding 200 μL of 0.5% sodium deoxycholate solution (Sigma-Aldrich, St. Louis, MO, USA) and counted by a plate count using MHS agar. Bacteria treated with miliQ water were used as a control.

Invasion assay The procedure described for the adherence assay with a few modifications was followed. After infection, the HeLa cells were washed with PBS and supplemented with fresh PBS containing 1% FBS and 150 μg/mL gentamicin (to kill the remaining viable attached bacteria). The cells were incubated for 1 h, washed twice with PBS, and lysed with 0.5% deoxycholate. The viable intracellular bacteria were quantitated using the plate count method. Results were expressed as the percentage of bacteria adhered in relation to the initial inoculum (%A) and the percentage of invasive bacteria in relation to the adhered bacteria (Ratio I/A) (21).

Quantitative PCR for adherence and invasion genes To evaluate the effects of the *Citrus* byproduct extracts on the expression levels of *cadF* (adherence, Table 2), *ciaB* (invasion, Table 2), and housekeeping gene *glyA* (housekeeping genes are involved in basic cell maintenance and, therefore, are expected to maintain constant expression levels in all cells and conditions), RNA was isolated from the bacteria as reported in previous studies (16,22) with some modifications. Briefly, *Campylobacter* cells were grown in MHB (42°C, 18 h, microaerobic conditions (CO_2 chamber, Mod 307; Thermo Fisher Scientific) in the presence of 75% of the MBC of the *Citrus* byproduct extracts until an $O.D._{600}$ of 0.5 (Spectrophotometer, mod 340; Sequoia Turner, Mountain View, CA, USA) was attained (10^7 CFU/mL) (23). Cells were collected from the cultures by centrifugation at 8,000×*g* for 5 min at 4°C (Eppendorf centrifuge, mod 5810R; Eppendorf, Pittsburg, PA, USA). Total RNA was extracted using the Trizol reagent (Sigma-Aldrich), according to the manufacturer's instructions (22,24). Expression levels of *ciaB*, *cadF*, and *glyA* was assayed by real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) using the One-Step iScript RT-PCR kit with SYBR green (Bio-Rad, Philadelphia, PA, USA), as described by the manufacturer. Reactions were performed in an iQ5 iCycler (Bio-Rad) after reverse transcription at 50°C for 10 min. The amplification protocol consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Threshold (C_t) values were determined using the iQ optical system software version 1.0 (Bio-Rad). The relative level of expression was calculated by the comparative threshold cycle method (ΔC_T) according to the BioRad Real-Time PCR Applications Guide (BioRad, 2006).

The ratios of the expression levels were calculated from the following equation:

$$\text{Ratio (reference/target)}=2^{\Delta CT}$$

where $\Delta C_T=C_T$ (reference gene)C_T (target gene).

This method analyzes the differences between the reference housekeeping gene *gly*A and the target genes (C_T values for each sample). The ΔC_T method is a variation of the Livak method (25). Although this method seems to be simpler than the Livak method, the results are essentially the same and the expression is normalized. The expression was determined for both treated and untreated cells.

Bioactive compound and antioxidant activity assays

***Total phenols and flavonoids*:** The total phenol content in the *Citrus* byproduct extracts was determined by the colorimetric Folin–Ciocalteu reagent using gallic acid as the standard (26). Briefly, 50 μL of hydrophilic *Citrus* byproduct extracts (adjusted to 1.0 mg/L) were added to 3 mL of deionized water plus 0.25 mL of Folin–Ciocalteu reagent (1 N, Sigma-Aldrich). After 5 min, 0.75 mL of 20% Na_2CO_3 solution was added and deionized water was added to reach a total volume of 5 mL. After incubation at room temperature for 30 min, the phenol concentration was measured spectrophotometrically at 760 nm and reported as mg of gallic acid equivalent (GAE) per g of dry weight.

The total flavonoid content was measured by the aluminum chloride reaction as reported by Zhishen *et al*. (27). *Citrus* byproduct extracts (1 mL) were mixed with 4 mL of deionized H_2O and 0.3 mL of $NaNO_2$ (5%). After 5-min incubation at room temperature, 0.3 mL of $AlCl_3$ (10%) was added and the solution was left to stand for 1 min. Then, 2 mL of 1 M NaOH was added with continuous stirring and

water was added to reach a total volume of 10 mL. Absorbance was measured at 415 nm using a UV spectrophotometer (mod. 6405; Jenwey, Staffordshire, UK). The total flavonoid content was expressed on a dry-weight basis as the milligrams of quercetin equivalents per gram (DW).

***Antioxidant activity*:** The radical scavenging activity was determined using the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) method and the Trolox-equivalent antioxidant capacity (TEAC, 28). $DPPH^{\bullet}$ was analyzed using the method described by Kedare and Singh (29) with some modifications. A stock solution of $DPPH^{\bullet}$ radical was prepared by mixing 2.5 mg with 100 mL of absolute methanol and adjusted to an $A_{515\ nm}$ of 0.7±0.02. Aliquots (3.9 mL) of $DPPH^{\bullet}$ radical were placed into test tubes, mixed with 100 μL of the extract, vortexed, and kept in a dark environment for 30 min. Absorbance was measured at 515 nm (mod. 6405; Jenwey). The radical scavenging activity was expressed as the inhibition percentage of the $DPPH^{\bullet}$ radical (% RSA). Antioxidant capacity of the *Citrus* byproduct extracts was determined from the content of $ABTS^{\bullet+}$ (2,2'-azino-bis [3-ethylbenzothiazoline-6-sulphonic acid]) using the TEAC. A working solution (1 mL of 7.4 mM ABTS with 1 mL of 2.6 mM $K_2S_2O_8$ adjusted to A_{734} 0.7) was prepared and an aliquot (2.97 mL) was mixed with 30 μL of *Citrus* byproduct extract or Trolox standard solution (dissolved in methanol). A_{734} was measured at 1 and 6 min during the reaction. Percentage inhibitions of $ABTS^{\bullet+}$ of the test samples were compared with those of the Trolox standard. Results were expressed as TEAC (μmol of TE/g of dry weight).

Statistical analyses All experiments were conducted at least three times in three separate experiments. Statistical analyses were performed using SPSS software (version 10.0, SPSS Inc., Chicago, IL, USA). Results were analyzed using the ANOVA test, followed by a post-hoc test using the multiple-comparison Turkey method. Spearman rank analyses were used to determine the correlation between the antioxidant activity of the extracts and the adherence and invasion of *C. jejuni* in HeLa cells. Differences between the means were considered significant if $p \leq 0.05$.

Results and Discussion

***Citrus* byproduct extracts reduce *C. jejuni* adherence and invasion** Addition of sub-inhibitory concentrations of *Citrus* byproduct extracts did not cause any significant reduction in the growth of any tested strain (11168, 5653, 238ip, and 180ip) than that achieved for untreated controls (Fig. 1). Therefore, 75% of the MBC was used for all subsequent assays (Table 1).

Strains tested without *Citrus* byproduct extracts showed different percentages of adherence (13.8, 5.4, 2.2, and 0.05% for strains 11168, 5653, 238ip, and 180ip respectively; Table 3). When the cells were treated with *Citrus* byproduct extracts, the adherence was reduced compared with that of the control ($p \leq 0.05$) in almost all cases, except when strain 180ip was treated with *C. limon* or *C. aurantium* (Table 3). The Spearman rank analysis indicated a correlation between the antioxidant activity (DPPH) and adherence (rho 0.569; p=0.026) for *C. jejuni* 180ip; however, this correlation was not observed for the other strains analyzed. Also, a correlation was detected between the antioxidant activity (TEAC) and adhesion for *C. jejuni* 11168 (rho 0.56; p=0.020) and *C. jejuni* 238ip (rho 0.58; p=0.027). In general, the ratio I/A bacteria varied among different strains (Table 3). Invasion was lower ($p \leq 0.05$) for all treatments compared with that observed for the control (Table 3). As observed for adhesion assays, I/A ratio showed a correlation with the antioxidant activity measured by DPPH in *C. jejuni* 180ip (rho=0.700; p=0.003). A similar correlation was observed between the antioxidant activity (TEAC) and the I/A ratio for *C. jejuni* 238ip (rho 0.644; p=0.009). For the case of the *C. jejuni* strain 5653, an inverse

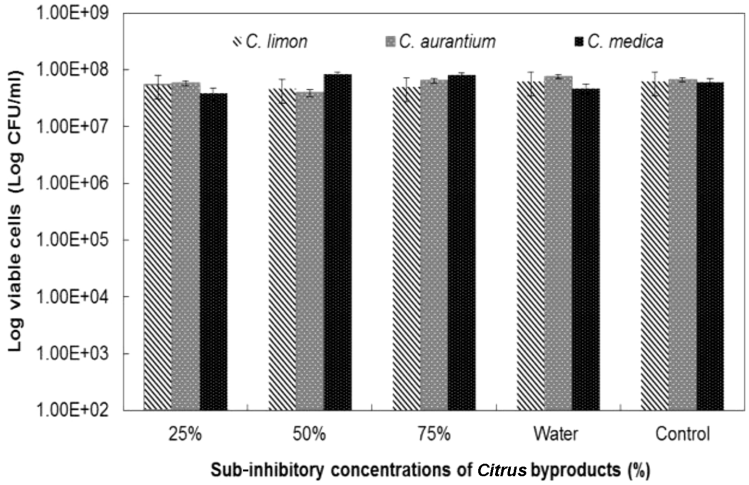

Fig. 1. Viable *Campylobacter jejuni* NCTC 11168 cells treated with sub-inhibitory concentrations of *Citrus* byproduct extracts. Concentrations used for *C. limon* were 168, 113, and 56 mg/mL (corresponding to 75, 50, and 25% MBC, respectively), for *C. auranticum* 244, 163, and 81 mg/mL (75, 50, and 25% MBC, respectively), and for *C. medica* 188, 125, and 63 mg/mL (75, 50, and 25% MBC, respectively).

Table 3. Percentage reduction of adherence and invasion in HeLa cells and I/A ratio by *C. jejuni* strains via effect of byproduct extracts

Strain	Citrus byproduct extract added	Reduction %		Adherence/ Invasion Ratio (% I/A)
		Adherence	Invasion	
C. jejuni NCTC 11168	Control	0	0	16.61
	C. limon	81.3	99.2	0.09
	C. aurantium	93.8	71.3	4.87
	C. medica	91	94.8	1.21
C. jejuni NADC 5653	Control	0	0	10.45
	C. limon	51.1	99	1.45
	C. aurantium	56.1	88.2	1.31
	C. medica	50.8	87.5	0.09
C. jejuni 238ip	Control	0	0	1.93
	C. limon	70	61.8	0.75
	C. aurantium	91.5	91.9	0.14
	C. medica	90.3	91.2	0.2
C. jejuni 180ip	Control	0	0	2.7
	C. limon	2.3	89.3	0.21
	C. aurantium	0	95.9	0.07
	C. medica	60.2	85.1	0.31

correlation was detected (rho=0.719; p=0.002) wherein a decreasing ratio I/A was observed with respect to the antioxidant activity of the extracts analyzed. Statistical analyses did not show differences between the effects produced by various extracts.

***Citrus* byproduct extracts reduce *cadF* and *ciaB* gene expression**

Expression levels of *cadF* (adhesion) and *ciaB* (invasion) genes were measured using the housekeeping gene *glyA* as a reference. Expression levels of *cadF* and *ciaB* were reduced ($p \leq 0.05$) in all cases compared with that for the control. The results of gene expression, varied depending on the analyzed strain and type of *Citrus* byproduct extract. No differences in gene expression were observed between the analyzed extracts, except for *cadF* in *C. jejuni* 180ip when treated with *C. limon* and *ciaB* in *C. jejuni* 238ip when treated with *C. aurantium* (Fig. 2).

Bioactive compounds and antioxidant activities of *Citrus* byproduct extracts

***Phenolic compounds*:** Differences ($p \leq 0.05$) were observed in the phenolic and total flavonoid contents of the extracts. In general, *C. aurantium* byproducts contained the highest levels of phenolic compounds (Fig. 3) and showed the maximum reduction of adherence (except for *C. jejuni* 180 ip). Total phenolic contents of *C. aurantium, C. limon,* and *C. medica* byproduct extracts were 92.0±4.8, 41.7±13.1, and 25.8±2.8 mg GAE/g of DW, respectively. Total flavonoid contents of the *Citrus* byproduct extracts exhibited differences, with the highest content being found in *C. aurantium* (161.09±0.2 mg QE/g), followed by *C. limon* and *C. medica* (63.97±0.3 and 29.29±5.6 mg QE/g, respectively).

***Antioxidant capacities of Citrus byproduct extracts*:** The highest DPPH radical scavenging activity ($p \leq 0.05$) was detected for the byproduct extract of *C. aurantium* (90.1±0.6%), followed by the extracts of *C. limon* (44.6±1.2%) and *C. medica* (43.8±0.3%) (Fig. 4). No significant differences were observed between *C. limon* and *C. medica* extracts. Antioxidant capacities differed ($p \leq 0.05$) among all *Citrus* byproduct extracts, with TEAC values of 15.8±0.4 μmol TE/g for *C. aurantium*, 19.8±0.1 μmol TE/g for *C. limon,* and 14.8±0.5 μmol TE/g for *C. medica* extracts (Fig. 4).

In this study, the effects of *Citrus* byproduct extracts on the adherence and invasion processes in *C. jejuni* were determined and

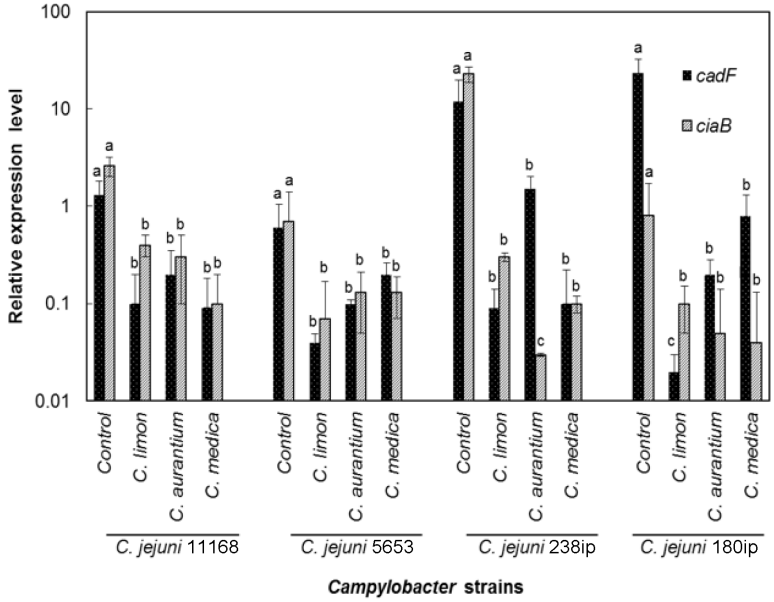

Fig. 2. Relative expression levels of *cadF* and *ciaB mRNA* in *C. jejuni* after treatments with *Citrus* byproduct extracts. Untreated cultures of each strain served as a control. Units are represented as $2^{\Delta CT}$ values. *glyA* housekeeping gene was used as a reference. Error bars show standard deviation. Letters (a, b, c) denote significant differences compared with their respective controls.

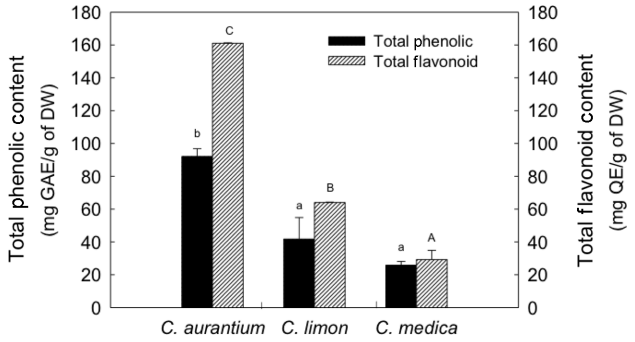

Fig. 3. Total phenolic and flavonoid contents of the three *Citrus* byproduct extracts.

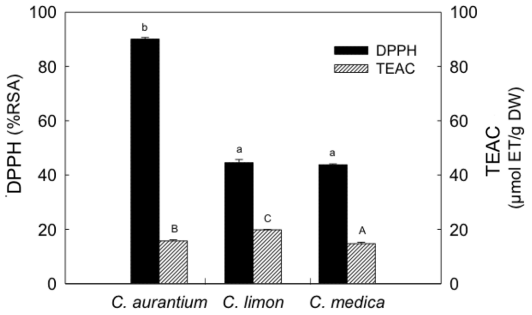

Fig. 4. Antioxidant capacities (DPPH and TEAC) of the three *Citrus* byproduct extracts. Different letters above bars represent signicant differences (p<0.05) between *Citrus* peel extracts, whereas same letters represent no significant difference.

their total phenolic contents and antioxidant capacities were analyzed. As key steps in the development of *C. jejuni* infection, adherence and invasion correlate with the severity of the symptoms and infection (30,31). *Campylobacter* strains used in this study showed differences in both processes, consistent with that reported in previous studies describing such variability (2,21). However, adherence and invasion abilities appeared to be correlated in almost all cases, in contrast to that reported by previous studies showing that the invasion process is independent of adherence (20,21). Invasion and adhesion could be influenced by the cell lines that are used for *in vitro* assays and genetic factors (2,20,21). In our experiments, the extents of adherence and invasion were reduced by treatment with *Citrus* byproduct extracts (Fig. 2). Similarly, Pogaèar *et al*. (32) reported a reduction in *C. jejuni* invasiveness in the human fetal small intestine cell line when *Alphinia katsumadai* extracts were used.

Genes *ciaB* and *cadF* encode the proteins involved in adherence and invasion. CiaB was first identified as an important secreted factor during invasion. CadF was the first described fibronectin-binding protein, playing a crucial role in the processes of adherence and colonization (4). However, the exact roles of these proteins during infection are not well understood (4). Our results showed that the expression levels of *ciaB* and *cadF* varied among the strains (Fig. 2).

We previously reported that the strain *C. jejuni* 238ip has low motility and the strain *C. jejuni* 180ip is non-motile (33). As motility plays an important role in adherence and invasion (5), both strains were expected to have low adherence and invasion abilities. As we anticipated, the non-motile strain showed lower adherence and adherence/invasion ratios than the motile strain. However, CadF and CiaB *mRNA* may be expressed at low levels in the non-motile *C. jejuni* cells and may contribute to low rate of adherence and invasion abilities. After treating the strains with *Citrus* byproduct extracts, the relative expression levels of *ciaB* and *cadF* were significantly reduced in all strains. These results agree with those reported by Lee *et al*. (34), who found that epigallocatechin gallate reduced the transcription of the major virulence genes in *E. coli* O157:H7. Likewise, Castillo *et al*. (16) reported a diminution of *flaA-B* expression in *C. jejuni* after treatment with *Citrus* extracts.

Phenolic compounds have been widely studied and their benefits to human health are well-known (8,35). Their protective activity has been mainly attributed to their free radical-scavenging abilities (36). *Citrus* byproducts, particularly peels, potentially represent a rich source of natural flavonoids. Although abundant in the plant kingdom, several flavonoids (e.g., avanones, avanone glycosides, and polymethoxylated avones) are unique to *Citrus* fruits (37,38). We analyzed the variations in the total phenolic content and antioxidant activity among the three *Citrus* extracts.

Gorinstein *et al*. (39) evaluated the total polyphenol content of *C. limon* and *C. sinensis* peels, reporting high content of total polyphenols in lemons. Ghasemi *et al*. (40) examined the total phenol and flavonoid contents in *C. aurantium* peels (232.2 mg GAE/g, 7.7 mgQE/g DM) and *C. limon* peels (131 mg GAE/g DM, 16.2 mgQE/g DM). Their reported values are higher than those reported in this study; however, the total flavonoid contents of byproducts from orange and lemon fruits were significantly higher in our study (41).

Previous studies described high variation in the total polyphenol contents of *Citrus* fruits, which could be because of the non-uniform peel compositions (40), differences in the extraction methods and solvents used, plant stress, geographical and climatic conditions, cultural practices, harvest time, and genotypic differences (42). We determined the antioxidant capacities of total phenolics and flavonoids in the *Citrus* byproduct extracts using two methods, the DPPH radical scavenging method and the TEAC assay (43,44). The two methods were chosen because *Citrus* extracts may contain varying proportions of phenolic components, which may have different antioxidant capacities. Significant differences in TEAC and %RSA were observed among the extracts; however, correlations were found between the DPPH (% RSA) assay for total phenolic (R^2=0.9448) and total flavonoid (R^2=0.939) contents. The DPPH assay showed the highest correlation with all the analyzed bioactive compounds.

Taken together, our results show that *Citrus* byproduct extracts could be good alternatives for devising new strategies to control *Campylobacter* infections. Further research should focus on food models and *in vivo* assays to understand the behavior of bacteria in

their natural environment.

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