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FOOD MICROBIOLOGY - RESEARCH PAPER

Campylobacter coli strains from Brazil can invade phagocytic and epithelial cells and induce IL-8 secretion

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Abstract

Campylobacter spp. have been a predominant cause of bacterial foodborne gastroenteritis worldwide, causing substantial costs to public healthcare systems. This study aimed to assess the invasion and pro-inflammatory cytokine production capacity of *Campylobacter coli* strains isolated in Brazil. A total of 50 *C. coli* isolated from different sources in Brazil were analyzed for their capacity of invasion in Caco-2 and U-937 cell lines. The production of pro-inflammatory cytokines was quantitatively measured in response to *C. coli*. All the strains studied showed invasion percentage ≥ 40% in polarized Caco-2 cells. In U-937 cells assay, 35 of 50 *C. coli* strains studied showed invasion percentage ≥ 50%. A significant increase in IL-8 production by infected U-937 cells was observed for 17.5% of the *C. co*li isolates. The high percentages of invasion in Caco-2 and U-937 cells observed for all studied strains, plus the increased production of IL-8 by U-937 cells against some strains, highlighted the pathogenic potential of the *C. coli* studied and bring extremely relevant data since it has never been reported for strains isolated in Brazil and there are a few data for *C. coli* in the literature.

Keywords Foodborne pathogens · Caco-2 and U-937 cell lines · Cytokines · Brazil

Introduction

Campylobacter has been a predominant cause of bacterial diarrheal disease in humans. According to Centers for Disease Control and Prevention (CDC), this bacterium causes an estimated 1.5 million illnesses each year in the USA being *Campylobacter jejuni* and *Campylobacter coli* the two main species reported in these cases [1–4]. However, in Brazil, the campylobacteriosis has been underreported and underdiagnosed, and there is a lack of sufficient data to access the incidence of this pathogen in the country [5–10].

Campylobacter species can be isolated from humans and animals, such as birds, mammals, reptiles, and, also from mollusks and protozoans. However, 50–80% of the reservoirs of this pathogen have been attributed to birds [11–13]. The transmission of this bacterial species occurs mainly via consumption and handling of improperly prepared poultry meat and consumption of unpasteurized milk [1, 14]. Campylobacteriosis is usually a self-limiting disease with symptoms such as fever, headache, abdominal cramps, and bloody diarrhea. However, in some cases, infections can lead to hospitalizations, post-infection sequelae, and death [15, 16].

Despite the high incidence of campylobacteriosis around the world, the knowledge of the mechanisms related to gut colonization and pathogenesis of *C. coli* species is more limited compared with that available for other foodborne pathogens [14, 17, 18]. Although the gut colonization is intrinsically related to the processes of adhesion and invasion to intestinal epithelium cells, this process is not completely clear for *C. coli* and the majority of the published studies were carried out with *C. jejuni* species [17, 19–21].

In general, human campylobacteriosis is a multifactorial process involving the interaction of bacteria to host epithelial cells and the response of the host immune system. Thus, in vitro models of human and animal cell culture and in vivo

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animal models have helped to clarify the mechanisms related to the infection process of emerging species such as *C. coli* [14].

In addition, studies have shown that *Campylobacter* is able to induce acute inflammatory enteritis [22–24] due to pro-inflammatory cytokine induction and subsequent recruitment of defense cells [25, 26]. In vitro experiments in human-derived cell lines [23, 26, 27] have shown that *C. jejuni* induced interleukin-8 (IL-8) which have also been found in stools of patients with campylobacteriosis [28, 29]. Moreover, secretion of IL-6 and tumor necrosis factor alpha (TNF-α) induced by *C. jejuni* and *C. coli* was also reported [23, 24, 30, 31].

Therefore, studies that can help to characterize and elucidate the pathogenesis of *C. coli* strains are of utmost importance since there is a paucity of data on this globally important pathogen and due to the fact that the majority of studies were performed with *C. jejuni* species.

The aims of this study were to assess the capacity of invasion to human colorectal adenocarcinoma cells (Caco-2) and human macrophages (U-937) of *C. coli* strains isolated in Brazil. Furthermore, the pro-inflammatory cytokine production was verified.

Material and methods

Bacterial strains

The 50 *C. coli* strains studied were selected from the collection of the *Campylobacter* Reference Laboratory of the Oswald Cruz Institute of Rio de Janeiro (FIOCRUZ) as described by Gomes et al [10]. These strains were isolated from human feces (12 strains), animals (15 strains), the environment (15 strains), and chicken meat (8 strains) from some cities in the Rio de Janeiro, São Paulo, and Minas Gerais States in the southeast region of Brazil between 1995 and 2011 (Table 1). Moreover, the *Salmonella typhimurium* ATCC 14028 and *Campylobacter jejuni* ATCC 33291 strains were used as controls in cell experiments.

The genus and species confirmation were performed by PCR to detect the specific regions of the 16S rRNA, *ceuE*, and *mapA* genes as described by Gomes et al [10].

Cell cultures

Caco-2 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, Arklow, Ireland) with 10% fetal bovine serum (Life Technologies, CA, USA) and 1% antibiotic/antimycotic (Life Technologies) in 75-cm^2 tissue culture flasks at 37 °C in a 5% CO_2 atmosphere until cell layers were confluent. The cells were seeded into 12-well tissue culture plates at a concentration of $1x10^5$ cells per well, and plates were incubated in a 5% CO_2 atmosphere at 37 °C for 12 days to provide the polarization of the cells [32, 33].

The U-937 were grown in Roswell Park Memorial Institute medium (RPMI) (Sigma-Aldrich) supplemented with 10% bovine fetal serum (Life Technologies) in a 5% CO_2 atmosphere at 37 °C until confluent. The cells were seeded into 24-well tissue culture plates at a concentration of 1×10^5 cells per well, and 10 nM of phorbol myristate acetate (PMA; Sigma) was added in the medium in order to differentiate monocytes in macrophages. The plates were incubated in a 5% CO_2 atmosphere at 37 °C for 24 h. Thereafter, the medium was changed, and the plates were incubated for additional 24 h without PMA in a 5% CO_2 atmosphere at 37 °C [33].

Caco-2 invasion assay

The *C. coli* strains were grown at 42 °C overnight and the inoculum was obtained as described by Gomes et al [10]. Bacterial growth was adjusted to an OD_{600} = 0.1 which correspond to approximately 8 $\log_{10}$ CFU/mL [20], and an aliquot was plated to verify the number of viable cells. Subsequently, the bacterial growth was centrifuged at 8000×*g* for 5 min and resuspended in 1-mL DMEM cell culture medium without antibiotic and/or fetal bovine serum. The OD_{600} = 0.2 was used to *S. typhimurium* ATCC 14028 control strain [34].

Each bacterial strain was tested in triplicate, and wells without bacteria were used as blank controls. The multiplicity of infection (MOI) of 100:1 was used to infect the Caco-2 cells with *C. coli*, and the bacterium-cell interactions occurred for 90 min in a 5% CO_2 atmosphere at 37 °C, as previously described by [35–40], with modifications. After incubation, the wells were washed with phosphate buffered saline (PBS 1X) and treated with 1 mL of the DMEM containing 30 μg/mL of gentamicin. The plates were incubated additional 90 min; the supernatant was seeded to ensure antibiotic activity and subsequently washed once with PBS 1X. After that, 1 mL of the DMEM with no antibiotics and no fetal bovine serum was added to each well that was incubated for 3 h in a 5% CO_2 atmosphere at 37 °C. The cells were washed with PBS 1X followed by a cell lysis with a 1% Triton X-100 solution for 5 min.

The CFU/mL were determined by serial dilutions in 0.8% saline solution and plating on Muller Hinton supplemented with 5% sheep's blood. The percentages of invasion in Caco-2 cells were determined by dividing the Log CFU/mL value after the cell lysis by log 10^7 (initial inoculum), and the results were multiplied by 100.

U-937 invasion assay

The inoculum was obtained according to what is described in item 2.3. After adjusting the optical density, an aliquot was

Table 1 Source, state, year, and IL-8 production induced of the 50 *Campylobacter coli* strains studied

Strains	Source	State	Year	IL-8 Concentration in U-937 (pg/mL)	IL-8 Concentration in Caco-2 (pg/mL)
CCAMP 771	Sewage	RJ	1995	2719.48	-
CCAMP 840	Sewage	RJ	1995	-	-
CCAMP 821	Monkey	RJ	1995	-	-
CCAMP 820	Monkey	RJ	1995	-	0.00
CCAMP 787	Sewage	RJ	1996	-	-
CCAMP 819	Sewage	RJ	1996	3004.62	-
CCAMP 765	Sewage	RJ	1996	2954.05	-
CCAMP 767	Sewage	RJ	1996	-	-
CCAMP 761	Sewage	RJ	1996	-	4.57
CCAMP 764	Sewage	RJ	1996	-	0.00
CCAMP 791	Monkey	RJ	1997	-	-
CCAMP 818	Sewage	RJ	1997	24.48	9.41
CCAMP 775	Sewage	RJ	1997	-	-
CCAMP 495	Human	RJ	1998	-	-
CCAMP 494	Human	RJ	1998	-	-
CCAMP 490	Human	RJ	1998	-	-
CCAMP 841	Monkey	RJ	1998	2490.68	0.00
CCAMP 502	Human	RJ	1999	-	0.07
CCAMP 498	Human	RJ	1999	-	0.00
CCAMP 975	Monkey	RJ	1999	83.66	-
CCAMP 726	Monkey	RJ	2000	-	0.00
CCAMP 503	Human	RJ	2000	-	-
CCAMP 834	Water	RJ	2000	-	0.23
CCAMP 595	Human	RJ	2001	-	-
CCAMP 667	Monkey	RJ	2002	2427.20	1.75
Cc 01	Human	SP	2002	4.51	-
Cc 10	Human	SP	2003	3504.63	0.00
Cc 04	Human	SP	2003	21.60	0.23
CCAMP 182	Monkey	RJ	2003	-	-
CCAMP 165	Monkey	RJ	2003	2185.62	0.00
Cc03	Human	SP	2003	1800.63	-
Cc05	Human	SP	2003	-	-
CCAMP 446	Monkey	RJ	2004	4023.50	-
CCAMP 463	Potable Water	MG	2004	17.57	4.51
CCAMP 469	Potable Water	MG	2004	11.76	-
CCAMP 464	Potable Water	MG	2004	-	-
CCAMP 394	Monkey	RJ	2004	-	-
CCAMP 769	Sewage	MG	2004	27.29	0.00
CCAMP 392	Monkey	RJ	2007	-	0.31
CCAMP 1010	Monkey	RJ	2007	2863.22	0.12
CCAMP 1000	Monkey	RJ	2007	23.50	-
CCAMP 1117	Monkey	RJ	2009	-	0.90
CCAMP 1062	Chicken wing	RJ	2010	22.60	0.11
CCAMP 1067	Chicken liver	RJ	2010	-	-
CCAMP 1063	Chicken gizzard	RJ	2010	-	-
CCAMP 1064	Chicken wing	RJ	2010	35.64	0.11
CCAMP 1066	Chicken liver	RJ	2010	25.06	-

Table 1 (continued)

Strains	Source	State	Year	IL-8 Concentration in U-937 (pg/mL)	IL-8 Concentration in Caco-2 (pg/mL)
CCAMP 1071	Chicken wing	RJ	2011	22.36	0.00
CCAMP 1075	Chicken liver	RJ	2011	22.44	-
CCAMP 1073	Chicken wing	RJ	2011	2279.46	-

MG Minas Gerais, *RJ* Rio de Janeiro, *RS* Rio Grande do Sul, *SP* São Paulo

plated to verify the number of viable cells, and 1 mL of stationary phase cells was centrifuged at 8000×*g* for 5 min and subsequently washed with PBS 1X for three times. The *C. coli* strains were opsonized with 20% of mouse serum (Sigma-Aldrich) at 37 °C for 15 min. After that, cells were centrifuged at 8000×*g* for 5 min and subsequently resuspended in 1 mL of RPMI cell culture medium without antibiotic and/or fetal bovine serum.

Each bacterial strain was tested in triplicate, and wells without bacteria were used as blank controls. The MOI of 100:1 was used to infect the U-937 cells with *C. coli*, and the bacterium-cell interactions occurred for 30 min in a 5% CO_2 atmosphere at 37 °C, as previously described by [40, 41], with modifications. After incubation, the wells were washed with PBS 1X and treated with 1 mL of the RPMI containing 30 μg/mL of gentamicin. The plates were incubated additional 90 min; the supernatant was seeded to ensure antibiotic activity and subsequently washed with PBS 1X. After that, 1 mL of the RPMI with no antibiotics and no fetal bovine serum was added to each well that was incubated for 3 h in a 5% CO_2 atmosphere at 37 °C. The cells were washed with PBS 1X followed by a cell lysis with a 1% Triton X-100 solution for 5 min.

The CFU/mL were determined by serial dilutions in 0.8% saline solution and plating on Muller Hinton supplemented with 5% sheep's blood. The percentages of invasion in U-937 cells were determined according to what is described in item 2.3.

Quantification of pro-inflammatory cytokine production in Caco-2 and U-937 cells

The production of pro-inflammatory cytokines IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 were quantitatively measured by the CBA Human Inflammatory Cytokines Kit (BD Biosciences, CA, USA) in response to *C. coli*. A total of 45 *C. coli* strains isolates from different year and sources were selected for this assay of which 24 *C. coli* strains showed different invasion percentages in U-937 cells and 21 *C. coli* strains showed different invasion percentages in Caco-2 cells.

The plates were prepared as described in item 2.2, and the inoculum was obtained according to what is described in item 2.3. To start the assays, each bacterial suspension was added to each well of plate with a MOI ratio of 100:1. Then, the plates were incubated in a 5% CO_2 atmosphere at 37 °C for 90 min for the bacterium-cell interactions [40, 41]. After the incubation, the supernatant of each well was collected, transferred to a properly labeled sterile tube, and stored in a freezer −80 °C until the quantification of pro-inflammatory cytokine assay.

In the assay day, the supernatant was processed using CBA Human Inflammatory Cytokines Kit according to the manufacturer's instructions and subsequently submitted to the BD LSRFortessa-1 flow cytometer.

Results

Caco-2 invasion assay

All the 50 *C. coli* strains studied (Table 1) showed invasion percentages higher than 40% in Caco-2 polarized cells (Fig. 1). Seventeen *C. coli* strains including nine isolated from humans (Cc 10 (85.7%), CCAMP 498 (80%), Cc 04 (78.9%), CCAMP 494 (75.7%), CCAMP 503 (75.7%), Cc 01 (74.6%), Cc 05 (74.6%), CCAMP 595 (74.6%), and Cc 03 (73.2%)), four isolated from the environmental (CCAMP 818 (83.2%), CCAMP 834 (77.5%), CCAMP 771 (76.7%), and CCAMP 787 (75.7%)), three isolated from animals (CCAMP 165 (88.9%), CCAMP 975 (74.6%), and CCAMP 182 (73.2%)), and one isolated from food (CCAMP 1071 (76.7%)) showed invasion percentages greater than or equal to the percentage showed by *S. typhimurium* ATCC 14028 (73.2%). The CCAMP 165 isolated from animal showed the highest invasion percentage (88.9%) when compared with the other *C. coli* strains studied (Fig. 1). Furthermore, 50% of the studied strains showed percentages of invasion greater than or equal to the percentage presented by *C. jejuni* ATCC 33291 (64.6%).

U-937 invasion assay

Thirty-six of 50 *C. coli* strains studied (Table 1) plus the *S. typhimurium* ATCC 14028 control strain showed the invasion

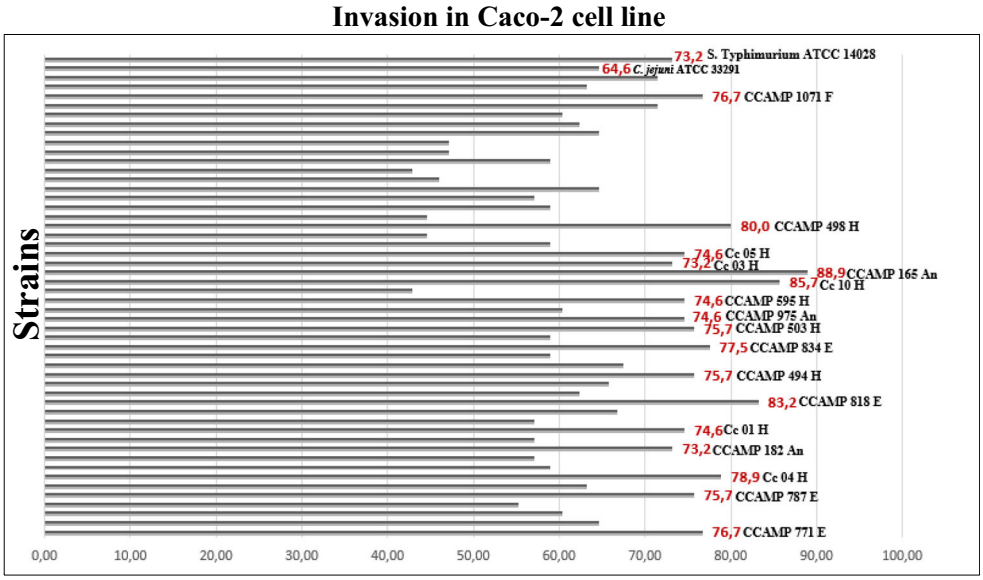

Fig. 1 Invasion percentages of 50 *Campylobacter coli* strains studied and the controls (*Campylobacter jejuni* ATCC 33291 and *Salmonella* Typhimurium ATCC 14028) in Caco-2 epithelial cells

percentages greater than or equal to 50% (Fig. 2). The CCAMP 667 strain isolated from animal showed the highest invasion percentage (76.7%) when compared with the others 49 *C. coli* strains studied. Seven *C. coli* strains including four isolated from human (Cc 03 (75.7%), Cc 01 (75.7%), Cc 04 (74.6%), and Cc 10 (73.2%)) and three isolates from animal (CCAMP 667 (76.7%), CCAMP 975 (73.2%), and CCAMP 841 (73.2%)) showed percentages of invasion greater than or equal to the percentage showed by *S. typhimurium* ATCC 14028 (73.2%). All the strains studied showed percentages of invasion greater than *C. jejuni* ATCC 33291 (30.4 %) (Fig. 2).

Quantification of pro-inflammatory cytokine production in CaCo-2 and U-937 cells

The quantitative analysis of pro-inflammatory cytokine production in U-937 cells in response to the presence of some *C. coli* strains after 90 min of bacterium-cell interactions showed high concentrations of IL-8 production (Table 1). The CCAMP 446 strain isolated from animal induced the highest IL-8 production in U-937 cells (4023.00 pg/mL). The CCAMP 819 strain isolated from the environmental and the strain Cc 10 isolated from human induced the IL-8 production in concentrations more than 3000.00 pg/mL. In

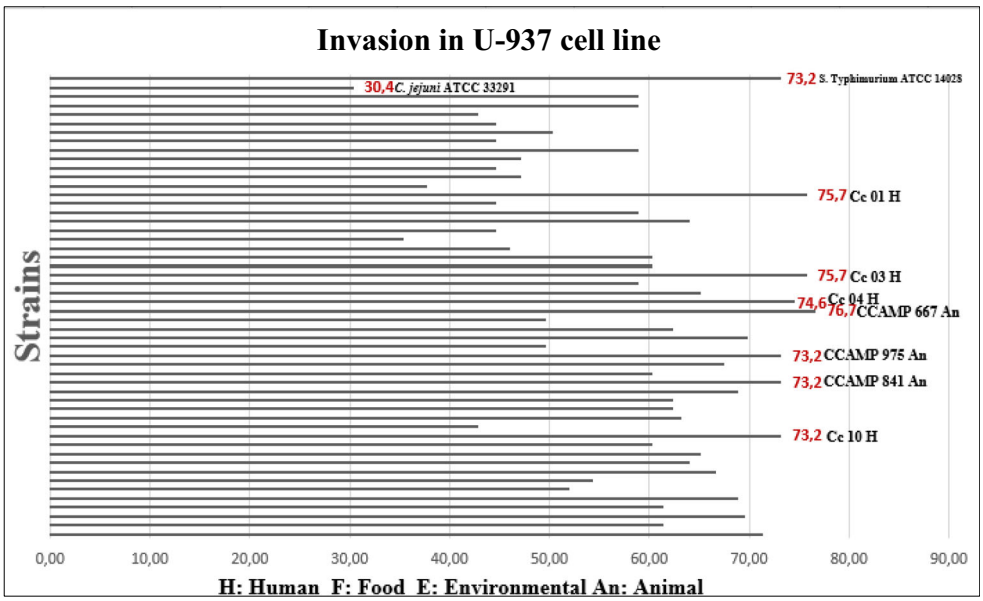

Fig. 2 Invasion percentages of 50 *Campylobacter coli* strains studied and the controls (*Campylobacter jejuni* ATCC 33291 and *Salmonella typhimurium* ATCC 14028) in U-937 macrophages

contrast, quantitative analysis of pro-inflammatory cytokine production in Caco-2 cells after 90 min of bacterium-cell interactions did not present significant values (Table 1).

The IL-12p70, TNF, IL-10, IL-6, and IL-1β cytokines analyzed against U-937, and Caco-2 cells did not show expressive values in response to the presence of some *C.coli* strains after 90 min of bacterium-cell interactions (data not shown).

Discussion

Campylobacter spp. have been a predominant cause of bacterial foodborne gastroenteritis worldwide, causing substantial costs to public healthcare systems [3, 4, 42]. Despite this, the exact mechanisms of *C. coli* infections are still not completely understood [14, 17, 18]. The gut colonization is intrinsically related to the processes of adhesion and invasion in intestinal epithelium, and the clinical manifestations of campylobacteriosis in humans have been directly linked to the virulence potential of different isolates together with the immunological predisposition of the host [43–48].

In the present study, we access the invasion and the inflammatory effect of *C. coli* strains, isolated from different sources in Brazil, in phagocytic and in intestinal human cells. Several models have been used to study the ability of *Campylobacter* to colonize and invade the gastrointestinal tract [30, 43]. Among them, Caco-2 epithelial cell model is the most commonly used being useful to mimic the behavior of *Campylobacter* in human gut [49, 50].

The invasion percentages in Caco-2 U-937 cells were greater than or equal to the percentage showed by *S. typhimurium* ATCC (73.2%), which has its invasion and pathogenicity mechanism extensively studied [51–54]. Specifically, the results herein obtained for *C. coli* strains isolated from humans suggested that these strains have a higher invasion capacity compared with non-clinical strains. Furthermore, 50% of the strains studied showed invasion percentages higher than or equal to the *C. jejuni* ATCC 33291 (64.6%) which has been often used in studies involving *Campylobacter* [55, 56].

To the generation of appropriate defense responses from the host, leukocyte recruitment to inflammatory sites is essential [57, 58]. In the invasion assay in U-937 human macrophage, 35 of 50 *C. coli* strains studied showed an invasion percentage higher than or equal to 50% what demonstrated a high survivability of the strains analyzed against human host defense cells (Fig. 2).

Of note, seven *C. coli* strains being four isolated from humans and three isolated from animals showed invasion percentages higher than or equal to the *S. typhimurium* ATCC 14028 (73.2%); in other words, strains isolated from clinical sources showed higher invasion percentages in human macrophages compared with strains isolated from food and the environment (Fig. 2). All the strains studied showed invasion percentages higher than the one presented by *C. jejuni* ATCC 33291 (30.4 %).

Although the molecular mechanisms of *Campylobacter* are poorly understood when compared with other foodborne pathogens, studies have shown that there are correlations between the severity of clinical symptoms and the invasiveness and survival capability of *Campylobacter* spp. [59–61]. Moreover, a great variability in invasion and survival capacity among *Campylobacter* isolates has been previously described [62–64].

It was shown that the invasion and survival capacity in both Caco-2 epithelial cells and U-937 phagocytic cells was strain-dependent [65–67]. Interestingly, the high invasion percentages observed in the present study was different from published data by Pan and colleagues and Zheng and colleagues [61, 65] that observed a remarkably low rate of adherence and invasion which reinforced the pathogenic potential of the *C. coli* herein studied and brings extremely relevant data that has never been reported for strains isolated in Brazil and contributed to the worldwide information once there is a few data for *C. coli* in the literature [27, 62–65, 67]. In previous studies of our research group, high growth and survival rates were also observed for the majority of those strains under different stress conditions such as tolerance to temperature variations, survivability in 7.5% of NaCl, survivability under acid, and oxidative stresses [10].

The high levels of pro-inflammatory cytokines in the early stage of *Campylobacter* infection suggest the development of an inflammatory cascade that is responsible for diarrhea during intestinal infection [68]. Adhesion and invasion to intestinal cells by *C. jejuni* induce secretion of IL-8, IL-1, IL-6, interferon-gamma (IFN-γ), TNF-α, and IL-4 among other cytokines that promote the recruitment of macrophages and neutrophils [25, 26]. In addition, studies have demonstrated that *Campylobacter* and *Lactobacillus acidophilus* interaction with Caco-2 cells induced the production of the pro-inflammatory cytokine interleukin IL-8, but this response was strain-dependent which could justify the absence of expressive values observed in this study [69–71].

The high concentration of IL-8 production observed in U-937 cells exposed for 90 min to the presence of *C. coli* strains selected from this study (Cc 03, Cc10, CCAMP 165, CCAMP 446, CCAMP 667, CCAMP 765, CCAMP 771, CCAMP 819, CCAMP 841, CCAMP 1010, and CCAMP 1073) demonstrated the immunogenic potential of some strains studied (Table 1). In addition, the results obtained in the pro-inflammatory cytokines analyses suggest that some studied *C. coli*, despite having high invasion percentages in both Caco-2 and U-937 cells, did not induce the production of the analyzed cytokines, which is an interesting fact as these strains have a previously demonstrated pathogenic potential, but they do not cause an immune response in the host.

It is important to mention that previous studies showed that *C. jejuni* can invade and evade cells without causing necrosis, apoptosis, cell lysis, and inflammation in the intestinal wall [72]. Thus, further studies to assess gene expression should be carried out in order to evidence more clearly the pathogenic potential of the studied strains.

In conclusion, the high percentages of invasion in Caco-2 and U-937 cells observed for all studied strains, plus the increased production of IL-8 by U-937 cells against some strains highlighted the pathogenic potential of the *C. coli* studied, a data that has not been reported before.

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Declaration

Conflict of interest The authors declare that they have no conflict of interest.

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