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ORIGINAL PAPER

Campylobacter fetus Induced Proinflammatory Response in Bovine Endometrial Epithelial Cells

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Abstract

Campylobacter fetus subsp. *fetus* is the causal agent of sporadic abortion in bovines and infertility that produces economic losses in livestock. In many infectious diseases, the immune response has an important role in limiting the invasion and proliferation of bacterial pathogens. Innate immune sensing of microorganisms is mediated by pattern-recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs) and induces the secretion of several proinflammatory cytokines, like IL-1β, TNF-α, and IL-8. In this study, the expression of IL-1β, TNF-α, IL-8, and IFN-γ in bovine endometrial epithelial cells infected with *C. fetus* and *Salmonella* Typhimurium (a bacterial invasion control) was analyzed. The results showed that expression levels of IL-1β and IL-8 were high at the beginning of the infection and decreased throughout the intracellular period. Unlike in this same assay, the expression levels of IFN-γ increased through time and reached the highest peak at 4 hours post infection. In cells infected with *S.* Typhimurium, the results showed that IL8 expression levels were highly induced by infection but not IFN-γ. In cells infected with *S.* Typhimurium or *C. fetus* subsp. *fetus*, the results showed that TNF-α expression did not show any change during infection. A cytoskeleton inhibition assay was performed to determine if cytokine expression was modified by *C. fetus* subsp. *fetus* intracellular invasion. IL-1β and IL-8 expression were downregulated when an intracellular invasion was avoided. The results obtained in this study suggest that bovine endometrial epithelial cells could recognize *C. fetus* subsp. *fetus* resulting in early proinflammatory response.

K e y w o r d s: bacterial infection, pathogenicity, virulence, pathogen-host interaction (MesH)

Introduction

The innate immune system senses microbial infections and triggers an immediate response to control pathogens' invasion. Microbial sensing is mediated by pattern-recognition receptors (PRRs), which include Toll-like receptors (TLR), Nucleotide-binding Oligomerization Domain (NOD), Leucine-rich repeat-containing receptors (NLRs), C-Type Lectin-Like Receptors, and Cytoplasmic Nucleic Acid Sensors. These receptors are important in innate and adaptative immune response because they identify Pathogen Associated Molecular Patterns (PAMPs) and determine the type of immune response required (Bryant et al. 2015). The innate immune response includes proinflammatory cytokines secretion, which recruits and activates phagocytic cells to eliminate the pathogenic microorganisms (Iwasaki and Medzhitov 2015).

The female reproductive tract's mucosal surface forms a physical and immunological barrier that can interact with sexually transmitted pathogens and spermatozoa. Therefore, innate immune mechanisms have an important role in maintaining its integrity (Amjadi et al. 2014). The cells in mucosal epithelia recognize pathogens and stimulate the underlying immune cells like macrophages, inducing an inflammatory reaction via cytokines' production, resulting in adaptive immunity activation. They also produce antimicrobial peptides that eliminate several bacterial and viral agents (Turner et al. 2014).

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Campylobacter fetus subsp. fetus is frequently isolated from the intestinal tract of asymptomatic cattle, goats, and sheep. In animals, *C. fetus* subsp. fetus exhibits a tropism for placental and reproductive tract tissues and is one of the major causes of sporadic and epidemic septic abortions (Viejo et al. 2001; Iraola et al. 2012). *C. fetus* subsp. fetus can attach in an irreversible way to bull spermatozoa and affect sperm quality (Cagnoli et al. 2020). The diseases produced by *C. fetus* subsp. *fetus* generate considerable economic losses, representing a significant problem in animal production (Mshelia et al. 2010). Heifers infected with *C. fetus* showed a light inflammatory reaction with few mononuclear and polymorphonuclear cells distributed diffusely beneath the epithelia of vagina and cervix, and moderate endometritis and salpingitis (Cipolla et al. 1994). Such light inflammation reaction can be due to the composition of the external membrane of microorganisms. *C. fetus* possesses lipooligosaccharides (LOS) instead of lipopolysaccharide (LPS) (Preston and Penner 1987; Moran et al. 2002). Also, it has a protein surface layer known as S-layer, which protects *C. fetus* against complement and opsonization-phagocytosis response; it also prevents recognition by host innate immune system (Blaser et al. 1987; Fogg et al. 1990; Blaser et al. 1993).

We have previously demonstrated the ability of *C. fetus* subsp. *fetus* to invade bovine endometrial cells (Campos-Múzquiz et al. 2019). This phenomenon was dependent on the viability of *C. fetus*, since dead bacteria could not invade this type of cells (in press). The ability of *C. fetus* subsp. *fetus* to invade endometrial cells raises new questions about the pathogen's mechanisms to infect these surfaces and induce reproductive diseases. The inflammatory response induced by *C. fetus* in bovine endometrium has not yet been entirely described; meanwhile, for other *Campylobacter* species, the induction of proinflammatory cytokines in epithelial cells has been described along with the benefits that this represents for tissue invasion (Al-Salloom et al. 2003; Zheng et al. 2008; Eucker et al. 2014). Hence in this study, the cytokine expression patterns induced in bovine endometrial epithelial cells by infection with *C. fetus* subsp. *fetus* were evaluated, to establish the role of inflammation in diseases produced by this species in the bovine reproductive tract.

Experimental

Materials and Methods

Bacterial strains and growth conditions. *C. fetus* subsp. *fetus* ATCC 27374 (Salama et al. 1995) was grown at 37°C for 48 h under microaerophilic atmosphere (85% N_2, 10% CO_2 and 5% O_2) on *Campylobacter* selective agar supplemented with 5% sheep blood. *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 was also grown on hyperosmolar Luria Bertani broth at 37°C for 12 h.

Endometrial epithelial cell culture. Epithelial cells from the endometrium were recovered using Skarzynski protocol (Skarzynski et al. 2015) with some modifications. The uterus was removed from three sacrificed cows 15 min after exsanguination at a slaughterhouse. Tissue was washed with Hank's solution supplemented with 1.6 mg/ml of gentamicin and transported to the laboratory in the same buffer solution on ice. The endometrium was cut and washed three times with phosphate-buffered saline solution pH 7.2 (PBS, NaH_2PO_4 1.9 mM, Na_2HPO_4 8.1 mM, NaCl 154 mM). Tissue pieces were treated with digestion solution (0.5 mg/ml collagenase type I from *Clostridium histolyticum*, 0.1 mg/ml DNAse, 100 µg/ml gentamicin, Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum) at 37°C for 2 h. A tissue debris-free supernatant was recovered and centrifuged at 4,000 × g for 10 min. The pellet was suspended in 5 ml of DMEM supplemented with 10% fetal bovine serum and filtered with a 40-µm strainer. Cells were placed on cell culture flasks with HEPES (25 mM) and antibiotic/antifungal solutions (penicillin G 10,000 U, streptomycin 5,000 µg, amphotericin B 12.5 µg). For fibroblast depuration, one-minute of trypsinization was performed daily for three consecutive days. Cell type was confirmed by immunofluorescence and RT-PCR (Campos-Múzquiz et al. 2019).

Invasion assays. Gentamicin protection assays were performed with *C. fetus* (multiplicity of infection – MOI – 100:1) and *S.* Typhimurium (MOI 50:1). An amount of 200,000 endometrial epithelial cells were cultured in a 25 cm^2 culture flask. Bacteria inoculum was diluted in DMEM, added to cultures, and incubated 2 hrs at 37°C. Following the incubation, the cell monolayers were washed three times with PBS and incubated with DMEM/gentamicin (10% fetal bovine serum, 25 mM HEPES, 30 µg/ml gentamicin) at 37°C. Cell lysates were recovered at 0, 2, and 4 h post-infection by adding 500 µl of Triton X100 (1%). Cell lysates (50 µl) of each infection hour was placed on *Campylobacter* selective agar supplemented with 5% sheep blood at 37°C for 72 h in an anaerobic chamber under low oxygen conditions (Oxoid Campy Gen, England). Cell lysates were treated with TRIzol (Invitrogen, USA) following the manufacturer's indication to recover cell and bacteria RNA. Three independent assays with three replications of each time were performed. For cytoskeleton inhibition, before the invasion assays, cell cultures were treated with cytochalasin D (3 mM) (Sigma-Aldrich, USA) for 30 min at 37°C. Then the invasion assays were made as described previously.

Intracellular survival evaluation by reverse transcription qPCR. Quantitative reverse transcription PCR using random primers and a commercial kit to synthesize complementary DNA (ProtoScript® First Strand cDNA Synthesis Kit, New England Biolabs) were used to evaluate the intracellular survival ability of *C. fetus* subsp. *fetus*. The cDNA was employed to quantify mRNA copies (RC) of *C. fetus* subsp. *fetus* and *Salmonella* Typhimurium (Power SYBR green, Fermentas). The primer sequences used in these assays for *C. fetus* subsp. *fetus* were: 5'-GGCAATATCATAGAAAATCCGTTATC-3' and 5'-TCCTGCTCTTTCATTTGCTT-3' these primers amplified a 161 bp fragment from fumarate reductase gene (*frd*A). The primers used for *S.* Typhimurium amplified a 110 bp segment of the *rpo*D gene (Botteldoorn et al. 2006). A standard curve was built with 1 ng, 100 pg, 10 pg, 1 pg and 100 fg RNA of *C. fetus* subsp. *fetus* ATCC 27374 or *Salmonella* Typhimurium ATCC 14028. The specificity of the PCR product was confirmed by high resolution melting curve.

Gene expression analysis of proinflammatory cytokines. RNA recuperated from invasion assays was used to synthesize cDNA using commercial kit and Oligo dT primers (ProtoScript® First Strand cDNA Synthesis Kit, New England Biolabs). A quantitative PCR (Maxima SYBR green, Thermo Fisher) was performed using the primers: TAF2 (5'-CATCTCCTGGAACCCAGAAA-3', 5'-GGCTGTTCTCCTCAATCTGC-3', 98 bp), β-actin (5'-AAATCGTGCGTGACATTAAG-3', 5'-GAGTACTTGCGCTCAGGAG-3', 341 bp) and GPDH (5'-GCCATCACCATCTTCCAGG-3', 5'-GGTAGTGGAGACCCCAGTGG-3', 115 bp), as reference genes; and IL-1β (5'-GAAAGAGACAACAAGATTCCTGTGG-3', 5'-GGTCTACTTCCTCCAGCTGCA-3', 108 bp), TNF-α (5'-CATCTACTCRCAGGTCCTCTT-3', 5'-GCAATGCGGCTGATGGT-3', 82 bp), IL-8 (5'-AGTACAGAACTTCGATGCCAATG-3', 5'-GTAAGCTTAACAATTTCTGAATTTTC-3', 127 bp), IFN-γ (5'-GGGTTTTTCTGGTTCTTATG - GC-3', 5'-GTCACTTTCATCTTTCCAATTCTT-3', 144 bp) as inflammatory genes. The specificity of the PCR product was confirmed by high resolution melting curve. The amplification efficiency (E) of each gene was calculated from the standard curves using the equation $E = (-1 + 10^{-1/\text{slope}}) \times 100$ (Livak and Schmittgen 2001). For gene normalization, we obtained a geometric average of the three reference genes (Vandesompele et al. 2002). The cytokine expression analysis was realized with treated cells with cytochalasin D to determine if the inhibitors could induce cytokine expression.

Statistical analysis. To analyze differences between gene expression and intracellular microorganisms, an F test was performed to establish the equality of variance of the data. A comparative CT method ($2^{-\Delta\Delta C}{}_T$) was used to calculate from each gene using the geometric average of the reference genes (Schmittgen and Livak 2008), and a Student's *t*-test was used to determine the difference between CT of treatment versus CT of control.

Results

Endometrial epithelial cell culture. The results obtained in primary cell cultures showed that the endometrial epithelial cells presented an epithelial-like appearance in the second week of incubation. In primary cell culture, the expression of Keratin 8 was confirmed by RT-PCR and immunofluorescence. A PCR product of 215 pb corresponding to a segment of the gene encoding for keratin 8 was obtained from RNA recuperated from cell cultures, and more than 90% of cells in the monolayer showed positive results to cytokeratin 18 (data not shown).

Invasion assays. In the intracellular survival assays, the results showed that viable *C. fetus* subsp. *fetus* decreased in number over time. At 0 h post-infection (p.i.) the average (~) colony-forming unit (CFU) were 22,408 CFU. At a second time (2 h p.i.), it decreased to ~ 1,316 CFU and 4 h p.i. there were ~ 233 CFU (Fig. 1); these results confirm that there was an intracellular bacterial cells reduction through the time ($p = 2.2e^{-16}$). In cells infected with *Salmonella* Typhimurium, the CFU increased at the end of the assay, indicating intracellular replication of bacteria ($p = 1.49e^{-10}$). At 0 h p.i., the average number of intracellular bacteria was ~ 42,150 CFU, 2 h p.i., CFU decreased to ~ 37,125 CFU, and 4 h p.i. bacteria proliferated, and their number increased to 72,925 CFU (Fig. 1).

To confirm *C. fetus* subsp. *fetus* intracellular survival ability, a bacterial mRNA quantification assay was

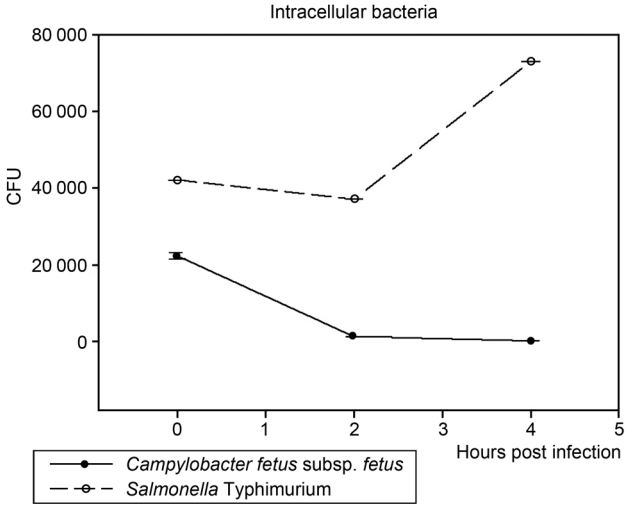

Fig. 1. *Salmonella* Typhimurium and *Campylobacter fetus* subsp. *fetus* invasion assays in endometrial cells. A gentamicin protection assay was performed to demonstrate that *C. fetus* subsp. *fetus* invades bovine endometrial epithelial cells but does not survive.

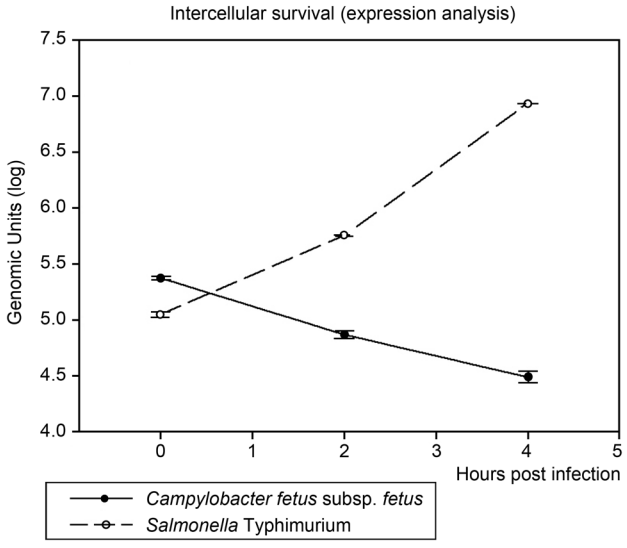

Fig. 2. Intracellular survival expression analysis of *Campylobacter fetus* subsp. *fetus* and *Salmonella* Typhimurium in bovine endometrial epithelial cells. An invasion assay was performed, and RNA was extracted from cells. The cDNA was synthesized using random primers, and a quantitative PCR was performed.

performed. For *C. fetus* subsp. *fetus*, *frd*A analysis (the constitutive gene) showed that the number of mRNA copies (genomic copies, GC) decreased significantly during invasion assays ($p = 0.002$). At 0 h p.i., the number of GC were ~5.3767 log, 2 h p.i., this value decreased to ~ 4.8724 log, and 4 h p.i., the value was ~ 4.4922 log (Fig. 2). The *rpo*D analysis in cells infected with *S.* Typhimurium showed that the number of GC of this constitutive gene increased through time ($p = 1.586e^{-06}$). At 0 h p.i., the number of GC was ~ 5.0526 log, 2 h p.i., it increased to ~ 5.7581 log, and 4 h p.i., it increased to 6.9379 log (Fig. 2).

Cytokine expression analysis. A gene expression assay was carried out to evaluate the expression of proinflammatory cytokines in endometrial cells infected with *C. fetus* subsp. *fetus*. Cells invaded by *C. fetus* subsp. *fetus* showed an early IL-1β high expression level (4.65-fold change at 0 h p.i.). Unlike cells invaded by *S.* Typhimurium, the IL-1β highest expression level was reached at 4 h p.i. (3.56-fold change). The cells infected with *C. fetus* subsp. *fetus* had the highest level of IL-1β (1.09 fold change, $p = 6.645e^{-05}$). The expression level of IL-1β decreased over time ($p = 7.492e^{-05}$) in cells infected with *C. fetus* subsp. *fetus*. At 0 h p.i., the fold change was 4.65, and it decreased to 3.78 2 h p.i., and this tendency was continued until 4 h p.i., where the fold change in expression was 1.09-fold. In cells infected with *S.* Typhimurium, the IL-1β expression levels showed an increment in time ($p = 0.0007$), at 0 h p.i, there was a 4-fold change value and increased to 3.56 at 4 h p.i. (Fig. 3).

The expression levels of IL-8 showed the same pattern in cells infected with *C. fetus* subsp. *fetus* and *S.* Typhimurium throughout the time. In both cases, IL-8 expression levels were high at the beginning of the infection and decreased through time ($p = 0.031$, $p = 5.127e^{-05}$, respectively). At 0 h p.i., the expression level of IL-8 in cells infected with *C. fetus* subsp. *fetus* there was a 3.41-fold change and 6.14-fold for cells infected with

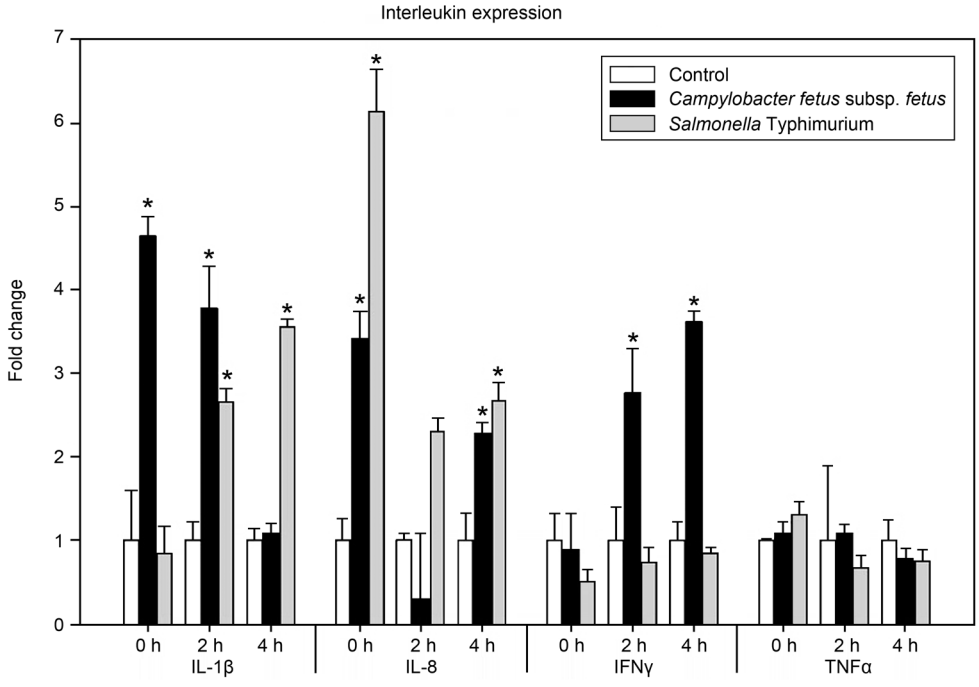

Fig. 3. Interleukin expression in bovine endometrial epithelial cells challenged with *Campylobacter fetus* subsp. *fetus* or *Salmonella* Typhimurium. Expression was analyzed with a $2^{-\Delta\Delta CT}$ and compared to control cells (no infected). A student *t*-test was performed to ΔCT (CT gene of interest – geometric media CT housekeeping genes) compared against non-treated cells; $^*p < 0.05$.

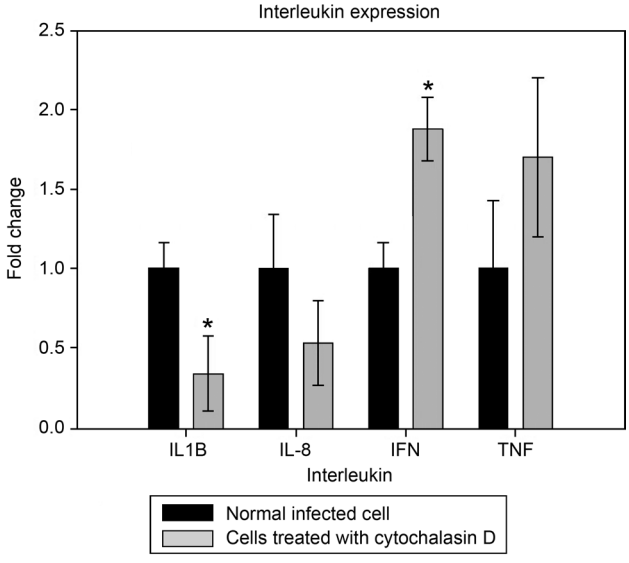

Fig. 4. Interleukin expression in bovine endometrial epithelial cells treated with cytochalasin D and challenged with *Campylobacter fetus* subsp. *fetus*. Expression was analyzed with a $2^{-\Delta\Delta CT}$ and compared to control cells (non-cytochalasin D-treated cells). A student *t*-test was performed to the ΔCT (CT gene of interest – geometric media CT housekeeping genes) comparing against non-cytochalasin D-treated cells; $^{*}p < 0.05$.

S. Typhimurium. Simultaneously, the expression levels of IL-8 were highest in cells infected with *S.* Typhimurium compared to cells infected with *C. fetus* subsp. *fetus* ($p = 0.015$). At 4 h p.i., for the expression level of IL-8 in cells infected with *C. fetus* subsp. *fetus,* there was a 2.28-fold change. For cells infected with *S.* Typhimurium, there was a 2.68-fold change (Fig. 3), so there was no difference in the expression levels between both treatments at this time (p-value = 0.4401) (Fig. 3).

The expression levels of IFN-γ in cells infected with *C. fetus* subsp. *fetus* showed a different pattern in comparison with IL-1β and IL-8. In this case, the expression level of IFN-γ increased through time ($p = 0.002$). At 0 h p.i., the expression level increased 0.89-folds, at 2 h p.i., it increased 2.77-fold, and at 4 h p.i., it reached a 3.62-fold increase (Fig. 3). In cells infected with *S.* Typhimurium, the expression levels of IFN-γ did not change throughout the time ($p = 0.367$). At 0 h p.i., the expression level was two times lower, 2 h p.i. it changed by 0.74, and 0.85 4 h p.i. Finally, the expression levels of TNF-α did not change either in cells infected with *C. fetus* subsp. *fetus* or *S.* Typhimurium, and there were no differences when compared to the control (uninfected cells) at any time during the assay.

To determine if *C. fetus* subsp. *fetus* intracellular invasion was necessary for the induction of IL-1β, IL-8, and IFN-γ, a cytoskeleton inhibition assay was performed, and cytokines expression was evaluated. In cells treated and infected with *C. fetus* subsp. *fetus*, the expression level of IL-1β was reduced by 0.34 compared with non-treated cells ($p = 0.005$). A similar result was observed, with IL-8 expression (0.53-fold change). Nonetheless, the difference was non-significant ($p = 0.105$). Finally, the expression levels of IFN-γ in cells treated with cytochalasin D and infected with *C. fetus* subsp. *fetus* showed an increment (1.90-fold change) in comparison with non-treated cells ($p = 0.021$) (Fig. 4).

Discussion

Pathogen-associated molecular patterns by PRRs upregulate the transcription of proinflammatory cytokines like IL-1β, TNF-α, and IL8 (Takeuchi and Akira 2010). In this study, endometrial epithelial cells showed a high expression of IL-1β and IL8 due to the infection with *C. fetus* subsp. *fetus*, suggesting that these cells could recognize some molecular patterns in this pathogen through their PRRs. These results agree with other studies that show the induction of proinflammatory cytokines by *Campylobacter* spp. infection (Wang et al. 2000; Arce et al. 2010b; Man et al. 2010; Yu et al. 2016). In the abovementioned studies, the authors used other types of cells as Caco-2, HEp-2, and HT-29 in their experiments. In this study, primary cell culture of endometrial cells was used, and the same phenomenon was observed during the infection. The early high IL-1β expression in cells infected with *C. fetus* subsp. *fetus* (4.65-fold change at 0 h p.i.) suggests an immediate immune recognition that might induce an acute inflammatory response. It could be used by *C. fetus* subsp. *fetus* to invade tissues. An invasion study in human trophoblast cells showed that in *C. rectus* there was a correlation between invasion and cytokine production (Man et al. 2010).

The IL-1β up-expression in bovine endometrial epithelial cells infected with *C. fetus* subsp. *fetus* was earlier than in *S.* Typhimurium (0 h p.i vs 4 h p.i respectively), and it decreased through time. Previously, a similar phenomenon was observed in a 4-week old chicken challenged with *S.* Typhimurium and *Campylobacter jejuni*. The peak of IL-1β expression in *C. jejuni*-infected chicks was at 20 h p.i., and in the *Salmonella*-infected chicks, it was at 48 h p.i. (Shaughnessy et al. 2009). A possible explanation for this could be that in the *C. fetus* genome, there are not virulence factors associated with immune suppression as in *S.* Typhimurium. Therefore, the induction of cytokines could be faster in *C. fetus* subsp. *fetus* in comparison with *S.* Typhimurium, which can modify the immune response (Kienesberger et al. 2014; Hu et al. 2017). Analysis of the present results also showed that the expression of pro-inflammatory cytokines in endometrial cells infected with *S.* Typhimurium took longer to reach the higher peak of expression, in comparison with cells infected with *C. fetus* subsp. *fetus*. Rolhion

et al. (2016) shown that *Salmonella* Typhi uses SpvD, an effector protein secreted through the type III secretion system, to avoid nuclear translocation of NF-*k*B transcriptional factor. Therefore, infected cells reduce proinflammatory cytokines secretion.

C. fetus subsp. *fetus* did not survive in endometrial cells in comparison with *S.* Typhimurium. In intracellular survival assays, the number of intracellular *C. fetus* subsp. *fetus* showed a reduction over time. This result was confirmed by two different methods, quantification of the number of transcripts (mRNA copies of constitutive genes) and CFUs. Considering that each method has different criteria to establish the number of viable microorganisms (gene expression and growth in specific media), we could say that reduction in the CFU number of *C. fetus* subsp. *fetus* was not due to the formation of viable non-culturable microorganisms but to the elimination of the pathogen. In the case of *S.* Typhimurium, the results showed a different pattern, indicating that the bacterium could survive and replicate inside the cells. This phenomenon has been previously reported (Campos-Múzquiz et al. 2019). These could be explained by *Salmonella* capability adaptation to intracellular niche (Larock et al. 2015) or by an immune stimulation that results in bacteria elimination. Furthermore, when *C. fetus* subsp. *fetus* internalization was inhibited, the IL-1β expression was halved. These results showed that *C. fetus* has been recognized intracellularly and that this recognition was required to induce higher inflammation and cellular mechanisms to eliminate the intracellular bacteria.

The results obtained in this study show a decrease in IL-1β expression through time. Stephenson et al. (2014) showed that *C. jejuni* flagellum binds to Siglec-10 of dendritic cells and increases IL-10 expression. This molecule has been described in human uterine cells (Sammar et al. 2016) but not in bovine endometrial cells. More studies are necessary to evaluate if the reduction of IL-1β was mediated by this molecule or only by infection control in the endometrial cells.

The pattern expression of IL-8 was like that of IL-1β, suggesting that *C. fetus* subsp. *fetus* infection promotes early neutrophil recruitment (Foley et al. 2012). In epithelial cell lines IPEC-J2 and IPI-2I, a high IL-8 expression was shown in response to *S.* Typhimurium LPS (Arce et al. 2010a), suggesting a prompt recognition by cells. The decrease of IL-8 also is due to an IL-10 increase described for IL-1β (Méndez-Samperio et al. 2002) or IFN-γ upregulation since it represses genes involved in leukocyte recruitment (Hoeksema et al. 2015). Moreover, when *C. fetus* subsp. *fetus* internalization was inhibited, the expression of IL-8 was not altered, indicating that the external sensing and not the bacteria's internalization induce polymorphonuclear recruitment (Takeuchi and Akira 2010).

Unlike our observation on IL-1β and IL-8 expression, IFN-γ showed an inverse pattern that increased over time in *C. fetus* subsp. *fetus* infected cells These results are related to the studies observed in INT-407 cells infected with *C. jejuni* in which IFN-γ peak was recorded at 12 hours post stimulation (Al-Amri et al. 2008). IFN-γ alters epithelial barrier function during inflammation by disrupting tight cell junctions and increasing permeability in polarized epithelial cells, which leads to cytosolic translocation of occludins and claudins (Bruewer et al. 2005). The increment of IFN-γ expression could be used by *C. fetus* subsp. *fetus* to invade submucosae.

In the presence of IFN-γ, *C. jejuni* was able to translocate across the monolayer more efficiently than in the absence of IFN-γ. This IFN-γ expression suggests also that epithelial cells from the endometrium might be associated with macrophage and adaptive immunity activation in campylobacteriosis (Hoeksema et al. 2015).

On the other hand, IFN-γ expression showed a slight increase when cells were treated with cytochalasin D. This could be due to a synergistic effect of *C. fetus* subsp. *fetus* sensing and *Campylobacter* toxins, for example, cytolethal distending toxin, which alters DNA (Rees et al. 2008). It has been reported before that cytochalasin D activates the p53 transcriptional factor, which controls DNA damage stress signaling (Rubtsova et al. 1998); however, more research is necessary to describe this phenomenon. The expression of IFN-γ in the cells infected with *S. enterica* subsp. *enterica* serovar Pullorum was non-significant. These results are not surprising since *Salmonella* possesses an immune response modulator IpaJ that decreases IFN-γ expression in macrophage cell line HD11 (Yin et al. 2018). Also, the principal sources of IFN-γ during *Salmonella* spp. infection are the neutrophils and NK cells (Pham and McSorley 2015).

In the cells infected with *S.* Typhimurium or *C. fetus* subsp. *fetus*, TNF-α expression did not change. A similar phenomenon was reported by Cronin et al. (2012) in bovine endometrial epithelial cells challenged with lipopolysaccharide. The same result was observed in bovine endometrial epithelial cells challenged with the danger-associated molecular patterns (DAMPs) (Healy et al. 2014). The TNF-α expression in these cells was possibly due to different conditions since the endometrium's primary function is blastocyst implantation (Kaneko et al. 2013).

In conclusion, the present study examined the proinflammatory response to *C. fetus* subsp. *fetus* in bovine endometrial epithelial cells. These bovine endometrial epithelial cells were able to recognize *C. fetus* subsp. *fetus* resulting in early proinflammatory response. Additionally, the internalization of the bacteria was necessary to induce IL-1β expression but not IL-8, suggesting the

importance of intracellular *C. fetus* subsp. *fetus* recognition. Their inability to survive inside of epithelial cells, the early induction of cytokines, and the upregulation of IFN-γ imply that the principal pathogenesis mechanism of *C. fetus* subsp. *fetus* in the uterine cavity is only to pass through the epithelium. This movement induces an inflammatory response that alters the tight junctions, as demonstrated in human HT-29/B6 and Caco-2 cells (Baker et al. 2010, Bücker et al. 2017).

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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