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RESPUESTA INMUNITARIA EN CÉLULAS ENDOMETRIALES BOVINAS INDUCIDA POR LA INFECCIÓN POR *Campylobacter fetus*

Tesis para obtener el grado de

DOCTORA EN CIENCIAS AGROPECUARIAS

PRESENTA

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Dedicatoria

A mi familia

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RESPUESTA INMUNITARIA EN CÉLULAS ENDOMETRIALES BOVINAS INDUCIDA POR LA INFECCIÓN POR *Campylobacter fetus*

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2. Campos-Múzquiz, L. G. Méndez-Olvera E.T., Castañeda-Ramírez A, Martínez-Gómez D. “Evaluación de la capacidad de invasión y sobrevivencia de *Campylobacter fetus* en células endometriales bovinas”. Rev. MVZ Córdoba (en revisión).
3. Campos-Múzquiz, L. G. Méndez-Olvera E.T., Martínez-Gómez D. *Campylobacter fetus* induce proinflamatory response in bovine endometrial epithelial cells. Central European Journal on Immunology (En revisión)

Resúmenes

1. Campos Muzquiz Lizeth Guadalupe, Méndez Olvera Estela Teresita, Martínez-Gómez Daniel1 Palacios Martínez Monika, Arellano Reynoso Beatriz, Castañeda Ramírez Alfredo. (2017) *Campylobacter fetus* invades and survives within bovine endometrial epitelial cells. "XL National Congress of Microbiology" Guadalajara, Jal. 2nd. – 5th. April 2017

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Introducción

Campylobacter es un género bacteriano en el cual se incluyen algunas especies causantes de zoonosis y enfermedades importantes para el sector pecuario, las cuales causan grandes pérdidas económicas debido a que provocan infertilidad y abortos en ovinos y bovinos, así como enfermedades gastrointestinales en aves.

Desde principios del siglo pasado, se identificó a *Campylobacter* como un patógeno importante en el sector pecuario. En 1906, Mc Fayden y Stockman identificaron un vibrio como causante del aborto epizoótico en ovejas, el cual fue aislado del moco uterino de una oveja y del contenido estomacal de su feto,por lo que llamaron a esta enfermedad aborto vibriónico. Este microorganismo no fue clasificado hasta el año 1919, cuando Smith y Taylor lo llamaron *Vibrio fetus* (Nachamkin *et al.*, 2008).

Posteriormente en 1947, Plastridge y colaboradores establecieron que existía una infección venérea transmitida por toros portadores. Las cepas del "vibrio" causante de esta infertilidad fueron descritas posteriormente por Florent en Bélgica en 1959 y se acordó su estatus de subespecie *Vibrio fetus* (ahora *Campylobacter fetus* subsp. *venerealis*) (Nachamkin *et al.*, 2008).

Campylobacter fetus se divide en tres subespecies: *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis* y *Campylobacter fetus* subsp. *testudinum*. La primera puede afectar a ovejas, al igual que algunas cepas de *Campylobacter jejuni*, ambas causando abortos tardíos, mortinatos y corderos débiles (Leedon y Spickler, 2013). *C. fetus* puede infectar al ganado bovino y otros ungulados. Esta bacteria ingresa al organismo a través de la ingestión de alimento contaminado y agua. Después de la ingestión, *C. fetus* subsp. *fetus* coloniza el intestino, y posteriormente puede presentarse una bacteremia, de esta forma el patógeno llega hasta la placenta provocando abortos (Nachamkin *et al.*, 2008). En cambio, *C. fetus* subsp.*venerealis* afecta únicamente a bovinos induciendo infertilidad, muerte embriónica temprana y abortos en bovinos. Esta enfermedad es conocida como Campilobateriosis Genital Bovina (CGB), Campilobacteriosis Venérea Bovina o Vibriosis Genital Bovina (OIE, 2017). Por otro lado, *C. fetus* subsp *testudinum* sólo se ha aislado de reptiles y genéticamente divergen de las otras dos subespecies (Gilbert *et al.*, 2016).

Según Van Bergen *et al.* (2005), las enfermedades en los animales pueden causar grandes pérdidas económicas debido a la muerte de los animales causada por la infección, a las estrategias que se deben de implementar cuando una enfermedad es introducida a una zona no endémica, a la pérdida en la producción, y a las barreras comerciales que se establecen cuando las fronteras son cerradas a la importación y exportación de animales y productos biológicos.

Dado que la CGB se presenta la mayor parte de los casos como una infección subclínica, no se sospecha de ella, hasta que los índices de fertilidad caen, produciendo perdidas económicas graves. En hatos infectados, las pérdidas en la reproducción son muy importantes y representan una gran pérdida financiera para los productores, sobre todo en el primer año de la infección, cuando los márgenes brutos pueden ser reducidos hasta en un 66 %. Una vez que la enfermedad se establece en un hato, los márgenes brutos suelen ser un 36% inferior a los de los hatos no infectados y la infección sigue siendo a menudo sin ser detectada, por lo tanto continúan las pérdidas de producción (Hum *et al.*, 2009). En México sólo existe un estudio donde se mencionan las pérdidas económicas debidas a esta enfermedad, en el cual se estiman 272 mil pesos por cada hato de 300 vacas (Flores y Ruiz, 1975).

La Organización Mundial de Sanidad Animal (OIE, 2017) describe a la CGB como una enfermedad notificable, en el Manual de las Pruebas de Diagnóstico y de las Vacunas para los Animales Terrestres 2017, en el capítulo 2.4.4 "CAMPILOBACTERIOSIS GENITAL BOVINA". En este manual se mencionan las vacunas para CGB, las cuales se incluyen antígenos somáticos, flagelares y capsulares, sin embargo aún se desconoce si estas proteínas son inmunogénicas, y hasta el momento no se ha estudiado la participación que pudiera tener la capa S en la evasión de la inducción primaria de citocinas de la respuesta inmune. Es por esto, que es necesario conocer las interacciones entre *C. fetus venerealis* y el bovino, a través de un entendimiento integrado del sistema inmune y la patogénesis para acelerar el desarrollo del conocimiento requerido para establecer estrategias de control efectivo, ya que, en opinión de W. Ivan Morrison (1999), director adjunto del Institute of Animal Health, Immunology and Pathology y consultor del IFPRI (Grupo Consultivo sobre Investigaciones Agrícolas Internacionales) "las oportunidades que presentan los adelantos en biotecnología solamente pueden explotarse debidamente si se entienden las características biológicas de los agentes patógenos escogidos como objetivo y las enfermedades que causan" y no se debe "perder de vista los grandes beneficios que se pueden derivar a largo plazo, de investigaciones estratégicas en patogénesis especialmente para la preparación de vacunas".

Por lo anterior, la identificación y caracterización de mecanismos de virulencia y estudio de la respuesta inmune, actualmente es un área vital en el desarrollo de metodologías para el control en muchos patógenos que afectan la producción animal.

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Resumen

El objetivo principal de esta tesis fue evaluar la producción de las citocinas proinflamatorias IL1β, IL8, IFNγ y TNF α en células endometriales bovinas, con el fin de establecer bases que permitan conocer la patogénesis de la vibriosis genital bovina.

Para esto primero se estableció un modelo de infección en el cual se utilizó cultivo primario de células epiteliales de endometrio bovino. Al desafiar estas células con *C. fetus* se logró establecer que la bacteria se internaliza en estas células, utilizando los filamentos de actina, no obstante no tiene la capacidad de replicarse dentro de las mismas.

Posteriormente, utilizando este mismo modelo se determinó por medio de la cuantificación relativa de la expresión de genes proinflamatorios, que *C. fetus* es reconocido por las células e induce una respuesta proinflamatoria. Además, demostramos que es necesaria la internalización de la bacteria para inducir esta respuesta en este tipo celular.

CAPÍTULO I. C*ampylobacter fetus* is internalized by bovine endometrial epithelial cells

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ORIGINAL PAPER

Campylobacter fetus is Internalized by Bovine Endometrial Epithelial Cells

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Abstract

Campylobacter fetus is an important venereal pathogen of cattle that causes infertility and abortions. It is transmitted during mating, and it travels from the vagina to the uterus; therefore, an important cell type that interacts with *C. fetus* are endometrial epithelial cells. Several virulence factors have been identified in the genome of *C. fetus*, such as adhesins, secretion systems, and antiphagocytic layers, but their expression is unknown. The ability of *C. fetus* to invade human epithelial cells has been demonstrated, but the ability of this microorganism to infect bovine endometrial epithelial cells has not been demonstrated. Bovine endometrial epithelial cells were isolated and challenged with *C. fetus*. The presence of *C. fetus* inside the endometrial epithelial cells was confirmed by the confocal immunofluorescence. *C. fetus* was not internalized when actin polymerization was disturbed, suggesting cytoskeleton participation in an internalization mechanism. To evaluate the intracellular survival of *C. fetus*, a gentamicin protection assay was performed. Although *C. fetus* was able to invade epithelial cells, the results showed that it did not have the capacity to survive in the intracellular environment. This study reports for the first time, the ability of *C. fetus* to invade bovine endometrial epithelial cells, and actin participation in this phenomenon.

Key words: bacterial infection, pathogenicity, virulence, pathogen-host interaction, infectivity

Introduction

Campylobacter fetus is a microaerophilic, Gram-negative bacterium that causes embryonic mortality, abnormal estrus cycles, reduced fertility and abortions in 5–10% of cases in cattle and sheep. *C. fetus* is divided into three subspecies: *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, and *C. fetus* subsp. *testudinum*. *C. fetus* subsp. *venerealis* is the etiologic agent of bovine genital campylobacteriosis, which causes infertility, abortions and embryonic death, and is mainly isolated from the genital tract (Nachamkin et al. 2008). *C. fetus* subsp. *venerealis* resides in the epithelial crypts of the prepuce and is transmitted to the cow by copulation or artificial insemination with the contaminated semen. On the other hand, *C. fetus* subsp. *fetus* is a commensal bacterium of the gastrointestinal tract of cattle and sheep. It can be associated with an infertility syndrome in cattle and abortions in sheep (Irons et al. 2004).

Although *C. fetus* is an animal health problem, little has been studied regarding its pathogenicity mechanisms. However, considering the pathogenesis of infection by this organism, it must possess characteristics that allow it to colonize or invade tissues and evade the immune system response. Several virulence factors, such as adhesins, secretion systems, and antiphagocytic layers, have been identified in the genome of *C. fetus* (Kienesberger et al. 2014). Nonetheless, it is still necessary to investigate *C. fetus* interactions with animal hosts.

When *C. fetus* reaches the genital tract of the cow, epithelial cells are the first cell type that it interacts with. These epithelial cells play important roles in innate immunity, such as acting as physical and immunological barriers, signaling the activation of the immune system through the production of cytokines and chemokines and inducing death in the infected cells (Farage et al. 2011).

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In previous works, it has been shown that *C. fetus* is able to adhere to and invade human epithelial cells; for example, Graham (2002) determined that different strains of *C. fetus* subsp. *fetus* isolated from human, and the *C. fetus* ATCC 27374 strain isolated from cattle, adhered to 41.3–87.3% and were internalized within 25.2–34.6% of INT 407 cells. Additionally, Baker and Graham (2010) demonstrated that *C. fetus* subsp. *fetus* can invade and translocate into Caco-2 cells. Chiapparrone et al. (2014; 2016) demonstrated the adhesion mediated by flagella to MDBK cells and adhesion to different parts of the sperm cells in *C. fetus* subsp. *venerealis*. Even though it has been described the ability of *C. fetus* to adhere to bovine cells, the ability of invasion to bovine endometrial epithelial cells of the bovine-adapted *C. fetus* strains has not yet been described. In this work, the ability of *C. fetus* to adhere and invade into bovine endometrial epithelial cells is evaluated.

Experimental

Materials and Methods

Bacterial strains and culture conditions. *C. fetus* ATCC 27374 has been described previously (Salama et al. 1995). *C. fetus* was grown on the *Campylobacter* selective agar supplemented with 5% sheep blood at 37°C for 48 h in an anaerobic chamber under the microaerophilic conditions. *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 has been previously described (Trüper et al. 2005). *S. enterica* Typhimurium was grown on the Luria Bertani agar and later inoculated in the hyperosmolar Luria Bertani Broth at 37°C for 12 h. *Escherichia coli* EPEC was donated by Dr. Jose Luis Puente from the Instituto de Biotecnología, Universidad Nacional Autónoma de México.

Primary endometrium epithelial cell cultures. Epithelial cells from the endometrium were recovered using the protocol by Skarzynski et al. (2000) with some modifications. The uterus was removed from five sacrificed cows 15 min after exsanguination. A piece of the uterus, 5 cm^2, was removed and washed three times in Hank's solution supplemented with 1.6 mg/ml gentamicin and transported to the laboratory in the same solution on ice. Serosa was removed from the tissue, and the rest was cut into small pieces (approximately 3 mm^2) and washed 3 times with pH 7.2 phosphate buffered saline (PBS) (NaH_2PO_4 1.9 mM, Na_2HPO_4 8.1 mM, NaCl 154 mM). Then, digestion solution (0.5 mg/ml collagenase Type I from *Clostridium histolyticum* (Sigma-Aldrich); 0.1 mg/ml DNAse (Thermo Fisher); 100 µg/ml gentamycin (Sigma Aldrich); in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (DMEM-S) was added and incubated at 37°C with oscillation for 2 h. The supernatant was recovered and centrifuged at 4000 × g for 10 min. The pellet was washed three times with DMEM:PBS (1:1). The pellets were resuspended in 5 ml of DMEM-S and filtered with a 40 µm strainer. The recovered cells were placed on the cell culture dishes with DMEM-S with 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and an antibiotic/antifungal (penicillin G 10 000 U, streptomycin 5 000 µg, and amphotericin B 12.5 µg). For fibroblast depuration, one-minute trypsinisation was performed every day for three days (Munson et al. 1988). The cell cultures were evaluated by RT-PCR and immunofluorescence to confirm that they were endometrial epithelial cells. First, the total RNA was extracted from cells using TRIzol following the manufacturer's methodology. Then, cDNA was synthesized using an AMV First Strand cDNA Synthesis Kit (New England Biolabs Inc.) following the manufacturer's instructions. The primers for Keratin 8 were as follows: forward 5'-CGTGTCAGAAATCTGAGACTGC-3' and reverse 5'-TGGTGGAGGACTTYAAGACC-3'. The PCR mixture was prepared with primers (40 nM), Master Mix (Fermentas) and 100 ng cDNA. The PCR conditions were as follows: pre-heat at 95°C for 5 min; 30 cycles of denaturalization at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 20 s. The PCR products were evaluated by electrophoresis in 2% agarose gels stained with ethidium bromide. For immunofluorescence, a polyclonal antibody against bovine cytokeratin 18 (Santa Cruz Biotechnology) was used in the cell cultures fixed in plates.

***C. fetus* PCR confirmation.** Three pairs of primers for the *asp*A, *gln*A, *glt*A and 16sRNA genes were designed. Before each assay, PCR was performed to confirm the purity of *C. fetus* cultures. The primer sequences and product sizes were as follows: *aspA*: F-5'-CCTATGACTTTAGGTCAAGAG-3', R-5'-TGTAG CTAGAGTACGGCAAG-3' (575 bp); *gltA*: F-5'-CGA-TATAGCGTGGCTAGCTG-3', F-5'-AGCGTGAGTAG ATCCTACG-3' (520 bp); *glnA*: F-5'-CTTCCGTTATC TCCATAAAGC-3', R-5'-GATGGTAGTTCTATAGA GGC-3' (649 bp); 16sRNA: F-5'-GAGATCACCAGGA ATACCC-3', R-5'-CACCTGTCTCAACTTTCTAGC-3' (351 bp). For these primers, the PCR conditions were a pre-heat of 95°C for 1 min, then 30 cycles denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s.

Cell adhesion assays. The adherence was evaluated by quantitative and qualitative methods. For quantitative evaluation, three independent adherence assays with three replicates were performed following the method described by Bacon et al. (2001). First, *C. fetus* was grown on the *Campylobacter* selective agar supplemented with 5% sheep blood for 48 h and harvested in PBS. *Escherichia coli* EPEC was used as an adhesion control and was grown in hyperosmolar LB for 12 h. The bacterial inoculum was determined by spectrophotometry and plating. The multiplicity of infection (MOI) was 1000:1. The infected cell cultures were centrifuged at 165 × g for 3 min to maximize bacteria-cell contact and incubated for 1 h at 37°C. The percentage of adhered bacteria was calculated by the formula [(CFU intracellular / CFU inoculum) × 100]. For visual interpretation, cells were fixed with methanol and stained with 100% Giemsa for 40 min.

Intracellular survival assays. Three independent assays with three replicates per time were performed as described by Elsinghorst (1994) with some modifications. First, *C. fetus* was grown on the *Campylobacter* selective agar supplemented with 5% sheep blood for 48 h and harvested in PBS. To prepare the inoculum, bacteria were quantified by spectrophotometry and diluted in DMEM to an adjusted MOI of 1000:1. Epithelial cells of the endometrium were seeded at 40 000 cells per well in a 48-well dish. After washing three times with PBS, the epithelial cells were infected. The plates were centrifuged at 165 × g for 3 min to maximize bacterial-cell contact and incubated for 2 h at 37°C. Following incubation, the monolayers were washed three times with PBS and incubated with DMEM supplemented with 10% fetal bovine serum, 50 mM HEPES and 30 µg/ml gentamicin. The plating of the samples of the medium confirmed the complete killing of the extracellular *C. fetus*. Bacteria were recovered at 0, 2, 4, 10 and 24 h post-infection (p.i.); supernatant medium was removed, and the intracellular bacteria were recovered by adding 1 ml of Triton X100 1% for 5 min, and the solution was homogenized and added to a microcentrifuge tube. Fifty microliters of each well were plated on the *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, catalogue number CN0025A). *S. enterica* Typhimurium was used as a positive control of infection because they can invade the bovine reproductive tract (Hall and Jones 1977).

Cytoskeleton inhibition assays. To establish the possible mechanism involved in *C. fetus* invasion in these epithelial cells, cytoskeleton inhibition assays were performed. Cytochalasin D (Sigma-Aldrich) was prepared as a 1 mM stock in dimethyl sulfoxide, and nocodazole was prepared as a 10 mM stock in dimethyl sulfoxide. Gentamicin protection assays were performed as described above, with some modifications. Forty thousand cells per well were plated in a 48-well dish. After washing three times with PBS, DMEM with cytochalasin D (3 mM) or nocodazole (30 mM) was added to the wells, and the cells were incubated for 30 min at 37°C. Then, bacteria were added, and the inhibitor concentration was kept at the half during the infection. The plates were centrifuged at 165 × g for 3 min to maximize the bacteria-cell contact and incubated at 37°C. *C. fetus* infection lasted for 2 h with an MOI of 1000:1 and *Salmonella* infection for 15 min with an MOI of 50:1. After infection, the inoculum was removed, and cells were washed three times with PBS and DMEM supplemented with 10% fetal bovine serum, Hepes 50 mM, and 30 µg/ml gentamicin. Cells were lysed with 50 µl of Triton X100 1% in each well and plated on the *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, catalogue number CN0025A).

Immunofluorescence Microscopy. Epithelial cells from bovine endometrium were infected as described above. At 0 and 2 h p.i., cells were washed three times with PBS and fixed with methanol for 4 min at 4°C. The fixed cells were washed three times with PBS and permeabilized by incubation in PBS containing 0.1% saponin for one min. Then, coverslips were incubated with 50 µg/ml phalloidin-FITC diluted with 1% dimethyl sulfoxide (DMSO) for 1 h at room temperature. The coverslips were washed three times with saponin 0.05% and PBS. After that, coverslips were incubated with primary antibodies (rabbit anti-*C. fetus*) diluted 1:1000 (saponin 0.05%, 5% horse serum, PBS) for an hour at 37°C. Afterward, the coverslips were washed three times with saponin 0.05% and incubated with secondary antibodies (goat anti-rabbit (US Biological) and diluted with Alexa Fluor 594 to 1:1000 for 30 min at room temperature. After three washes with 0.05% saponin, coverslips were mounted onto glass slides. Images were acquired with a Zen Zeiss LSM800 fluorescence microscope.

Statistical analysis. Each assay was subjected to a Shapiro-Wilks test and a Welch's test. Then, to determine the time at which the intracellular bacterial count was different in each treatment, Student's T-test was used.

Results

***C. fetus* adheres to epithelial cells of bovine endometrium.** To confirm that *C. fetus* could adhere to epithelial cells from the endometrium, an adherence assay was performed. First, endometrial epithelial cell cultures were established from cells isolated from uterus tissue obtained from slaughtered cows. The cells established a monolayer until the second week of incubation when these cells presented a polygonal epithelial-like form (Fig. 1A). These cells were used until the seventh passage since later splits presented different morphologies and diminished cell proliferation rates. For cell type confirmation, cytokeratin 18 from epithelial cells was immunostained with Alexa 488 (Fig. 1B), and RT-PCR for keratin 8 was performed (Fig. 1C). The results obtained

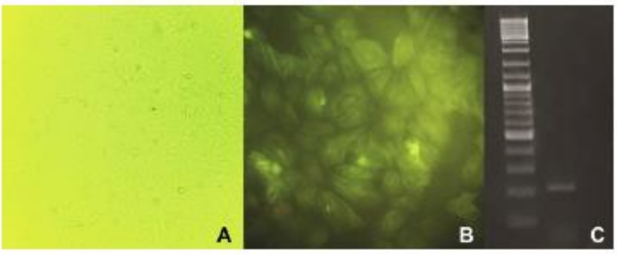

Fig. 1. Epithelial cells from bovine endometrium maintained in DMEM supplemented with 10% fetal bovine serum.

A) Normal appearance of the epithelial-like cells (20×). B) Immunofluorescence. Cytokeratin 18 of epithelial cells of bovine endometrium stained with Alexa 488 (green) (40×). C) The results of RT-PCR for keratin 8 on 2% agarose gel after staining with ethidium bromide. In left lane: DNA ladder (Thermo Fisher Scientific); right lane: the amplicon of keratin 8 (215 pb).

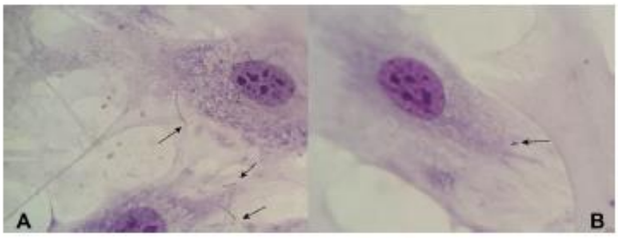

Fig. 2. The adherence assay. Epithelial cells from bovine endometrium were challenged with *C. fetus* (A) or *E. coli* (B) for 1 h, fixed with methanol and stained with Giemsa (100×). The arrows show the adhered bacteria.

show that cells in culture corresponded to endometrial epithelial cells. In the adherence assays, the average percentage of epithelial cell-adhered bacteria was 0.13%. Adhered *C. fetus* was observed by microscopy (Fig. 2A). *E. coli* EPEC showed a typically localized adherence pattern (Fig. 2B). These results confirm that *C. fetus* can adhere to endometrial epithelial cells.

***C. fetus* invades bovine endometrial epithelial cells.** After the adherence capacity of *C. fetus* was confirmed, a gentamicin protection assay was carried out to determine the intracellular survival of *C. fetus* in epithelial cells from bovine endometrium. The results obtained with MOI of 1000:1 showed a decrease in the number of *C. fetus* viable cells. At 0 h p.i there were an average (~) of 3795 CFU, at 2 h p.i they decreased to ~ 163 CFU, at 4 h there were ~ 36 CFU and 10 h p.i the organisms didn't grow. No viable cells were recovered at 24 h p.i., suggesting that the bacteria have the capacity to enter the cells, but it might not survive inside epithelial cells of the endometrium (Fig. 3). *Salmonella* invaded the bovine endometrial epithelial cells used in this study. At 0 h, the bacteria were present inside the cells ~ 8430 CFU, and at 4 h they started to replicate, increasing to ~ 14 585 CFU showing normal intracellular infection behavior in bovine epithelial cells (data not show). The presence of *C. fetus* inside the cells was confirmed by confocal microscopy. Before immuno-

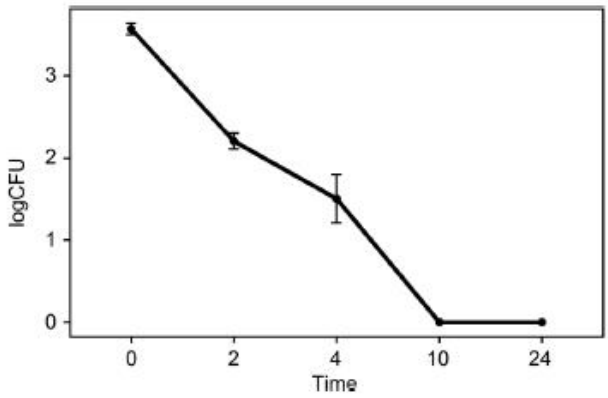

Fig. 3. The intracellular survival assay. Epithelial cells of endometrium of bovine were infected with *C. fetus* ATCC 27374 (a MOI of 1000:1). The intracellular bacteria were recovered and plated on the Campylobacter selective agar supplemented with 5% of blood. Average log CFU are shown at 0, 2, 4, 10 and 24 h p.i.

fluorescence, rabbit anti-*C. fetus* serum was adsorbed to avoid unspecific labeling and tested by Western blotting (Fig. S1, supplemental material). Additionally, the secondary antibody specificity and anti-rabbit HRP antibodies were tested by Western blotting. The results obtained in the Western blots, with a total bacterial protein extract and total cell protein extract, showed that adsorbed anti-*C. fetus* antisera recognized only antigens in *C. fetus* and not in epithelial cells, and the anti-rabbit antibodies did not recognize antigens in bacteria or epithelial cells (Fig. S1, supplemental material). The confocal fluorescence at 2 h p.i. showed the

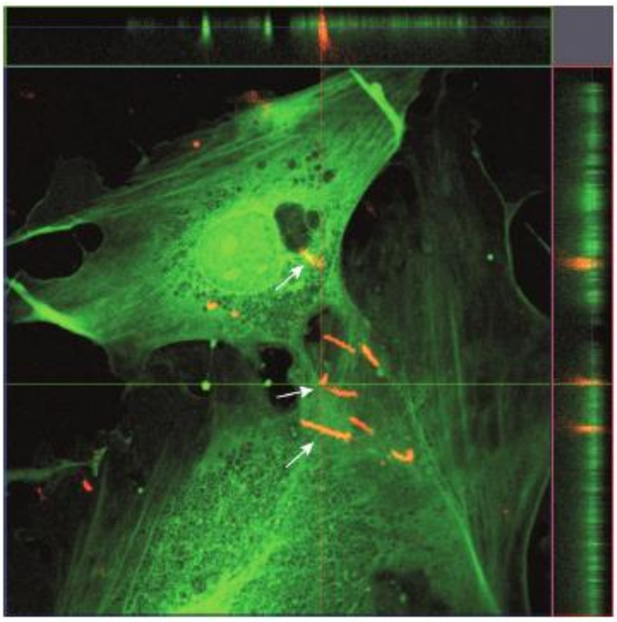

Fig. 4. Confocal differential fluorescent staining of internal *C. fetus* ATCC 27374 on the infected epithelial cells of bovine endometrium. Epithelial cells were grown on coverslips and infected with *C. fetus* ATCC 27374 at a MOI of 1000:1. Cytoskeleton was stained with phalloidin-FITC (green), and the bacteria with Alexa 594 (red) 2 h p.i. White arrows show intracellular *C. fetus* (70×).

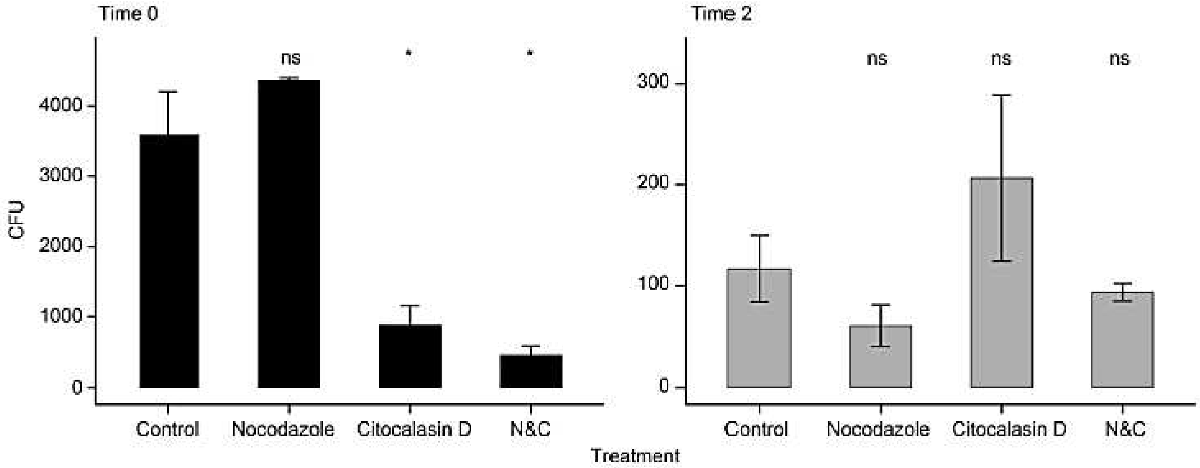

Fig. 5. The cytoskeleton inhibition assay. Epithelial cells of endometrium of bovine were treated with cytocalasin D or nocodazole before and during infection. The cells were infected with *C. fetus* ATCC 27374 (a MOI of 1000:1). The intracellular bacteria were recovered and plated on the Campylobacter selective agar supplemented with 5% of blood. Average log CFU are shown at 0 and 2 p.i. T-test was performed. All treatments were compared to the not-treatment control, *($p < 0.001$).

intracellular bacteria, demonstrating that *C. fetus* can invade epithelial cells from bovine endometrium but is not able to proliferate inside of them (Fig. 4).

***C. fetus* uses actin to invade bovine endometrial epithelial cells.** Internalization of *C. fetus* was modified when the cytoskeleton of the endometrium epithelial cells was altered. Treatment with cytochalasin D decreased the capability of internalization of *C. fetus* (~873 CFU). There was no change in *C. fetus* internalization when using nocodazole (~4330 CFU) suggesting that actin plays an important role in this process (Fig. 5).

Discussion

The invasion of *C. fetus* in some human cell lines, such as Hep-2 and Caco-2 has already been described (Konkel and Joens 1989; Baker and Graham 2010). Although these results suggest the ability of this microorganism to invade cells, the interaction with endometrial epithelial cells, the first type of cell that interacts with *C. fetus*, has not been described. In this work, an intracellular survival assay including gentamicin protection and immunofluorescence showed that *C. fetus* can invade the bovine endometrial epithelial cells, this result relates with the ones reported in the literature, which shows that *Campylobacter* spp. is able to invade cells (Konkel and Joens 1989; Graham 2002; Baker and Graham 2010).

The use of primary cultures for the evaluation of virulence of different pathogens has been widely reported. Most studies investigating the relationship between the host and bacteria, tend to focus on the cell types that comprise the biological barriers, signaling events within the host and the virulence factors of the pathogen, which are involved in the initial phase of the disease (Benjamin et al. 2011). In this work, a primary culture of bovine endometrial epithelial cells was established, considering that these cells could be the first in contact with *C. fetus*. The primary cultures showed a positive reaction against cytokeratin 18. The expression of this protein has been described in bovine endometrial epithelial cells (Haeger et al. 2015).

Cell culture models can compartmentalize and define the broad range of molecular mechanisms that underlie strategies of microbial virulence such as host receptor ligand binding and invasion mechanism. These mechanisms have not been identified in *C. fetus*; however, in this work, its ability to adhere to bovine cells was shown. The pathogenicity factors that could mediate this adhesion could be diverse. McSweegan and Walker (1986) described the role of lipopolysaccharide as a molecule that allows the adhesion of *C. jejuni* to INT407 cells. Monteville et al. (2003) identified a *C. jejuni cad*F homologous gene in *C. fetus*, which encodes for an outer membrane fibronectin binding protein (Accession No. Nz_CP00880). This protein in *C. jejuni* binds to fibronectin (Konkel et al. 1997), which is expressed on the epithelial cells of the endometrium (Mularoni et al. 1992). In *C. fetus* Moolhuijzen et al. (2009) have identified the *PEB1* gene, which participates in adhesion in *C. jejuni*. It has been described that *C. fetus* has a protein coat that may interfere in the contact with other cells (Yang et al. 1992). However, in this study, *C. fetus* was able to invade epithelial cells, so it is possible that the protein layer does not interfere, or it may exist some other mechanism, not yet described, for this bacterium that allows it to adhere the epithelial cells.

Intact epithelial surfaces are a highly effective barrier to evade invasion by pathogens. A capability to disrupt intact epithelial surfaces is an important characteristic for many specialized bacterial pathogens. In this work *C. fetus* showed the capability to invade cells; however, internalization mechanisms have not been described for these species. *C. fetus* possesses a type IV secretion system (Kienesberger et al. 2014), which is used by many pathogens for substrate translocation, for example in *Bartonella henselae* this system translocates BepC and BepF, the proteins factors that trigger invasome-mediated internalization (Truttmann et al. 2011). In *C. fetus,* Ali et al. (2012) identified a CiaB homologous, which in *C. jejuni* is translocated through flagellum and is required for internalization (Konkel et al. 1999). More research is required in order to identify the virulence mechanisms that mediate cellular invasion.

The previous reports have demonstrated that *C. jejuni* make use of microtubules to invade epithelial cells (Oelschlaeger et al. 1993; Hu and Kopecko 1999). In this work, it was observed that when actin polymerization was inhibited, *C. fetus* could not be internalized in the same way as the control, suggesting that actin plays an important role in the internalization mechanism, too. Moreover, Baker and Graham (2010) showed that cytochalasin D treatment on Caco-2 epithelial cells was not able to inhibit *C. fetus* internalization. Those results differ from the ones obtained in this work, the treatment with cytochalasin D of our primary culture did not inhibit totally the invasion of *C. fetus*, it only reduced it. This difference could be explained by the treatment conditions with cytochalasin D or by the incubation time of the bacteria in the cellular infection assays.

Although in this work was shown that *C. fetus* had the ability to invade cells, its intracellular survival was minimal (at 10 hours post infection the number of the intracellular bacteria had decreased significantly). Treatment of the cells with nocodazole, a drug that inhibits endosome-lysosome fusion (Funato et al. 1997), did not help *C. fetus* to survive inside the cell. This suggests that their inability to persist within cells could be explained by metabolic adaptations for the intracellular environment and not necessarily by lysosomal degradation. The intracellular bacteria require the metabolic adaptations to remain alive in the harsh intracellular environment. For example, *S. enetrica subsp.* Typhimurium shows upregulation of glycolysis and the Entner-Doudoroff pathway during the vacuolar stage (Eisenreich et al. 2015). *Campylobacter* spp. does not ferment carbohydrates because it lacks phosphofructokinase and essential enzymes for the Entner-Doudoroff pathway, and its principal source of carbon are amino acids (Kelly 2008). *Legionella pneumophila* also lacks the glycolysis pathway and uses amino acids as a carbon source, but unlike *Campylobacter*, it uses the Entner-Doudoroff pathways as an important carbon source (Eisenreich et al. 2015).

Another explanation that could help understanding why in the intracellular survival assay *C. fetus* was internalized and eliminated from the cells, is the metabolic reprogramming, which does not allow *C. fetus* to grow on agar plates after having gone through the intracellular stage. In *C. jejuni*, during its intracellular stay, the respiration is reprogrammed, favoring the use of fumarate and reducing the expression of enzymes of aerobic respiration (Liu et al. 2012). In this way, when bacteria are recovered in artificial media, their growth is reduced. This explanation could be the least likely, however, in the work done by Watson and Galán (2008) this phenomenon was found.

The invasive nature of *C. fetus* has been well established *in vivo* in clinical veterinary situations and *in vitro* with human intestinal epithelial cells assays. Baker and Graham (2010) showed the translocation of *C. fetus* through the barriers of intestinal epithelial cells with the culture of Caco-2 cells. Louwen et al. (2012) also showed a similar phenomenon with *C. jejuni* in Caco-2 cells. Therefore, the short permanence of viable *C. fetus* in the endometrial culture cells could be interpreted as normal, considering that the only objective of *C. fetus* is to be translocated.

There are many studies on *Campylobacter* spp. invasion capability (Mooney et al. 2003; Watson and Galán 2008), however, this report presents the ability of *C. fetus* to invade bovine endometrium epithelial cells. This could be used in future work as a bovine infection model and be an important element for understanding the pathogenicity mechanisms of *C. fetus*.

C. fetus could have been originated as a pathobiont in humans and jump to bovine as their host, generating adapted strains. The bovine strains contain in their genome the unique accessory genes (virulence factors) not seen in human strains (Iraola et al. 2017). This would explain why *C. fetus* can persist in the intestine of humans (Lastovica and Skirrow 2000) and may be associated with infertility and sporadic abortions in cattle and sheep (Irons et al. 2004). The *C. fetus* strain used in this work was isolated from a clinical case of an aborted calve, and it is known to cause abortions in cattle (Smith and Taylor 1919; Véron and Chatelain 1973). Graham et al. (2002; 2010) used this strain in some invasion assays using human cell cultures.

In conclusion, this work showed that *C. fetus* adheres and invades bovine endometrial epithelial cells. Gentamicin protection assays and fluorescence microscopy suggest that *C. fetus* can survive inside cells for only a few hours (4 h). Additionally, *C. fetus* is internalized using an actin-dependent mechanism in this cell type. All knowledge generated in this area will serve to propose and develop new strategies for the control of pathogens.

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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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Supplementary materials are available on the journal's website.

CAPÍTULO II

Campylobacter fetus induce proinflamatory response in bovine endometrial epithelial cells

Campylobacter fetus induce proinflamatory response in bovine endometrial epithelial cells

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Abstract

The innate immune system can recognize microbial agents and triggers an immediate response to control infection. Microbial sensing is mediated by pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). The PRRs-PAMPs interaction induces the secretion of the proinflammatory cytokines IL-1β, TNF-α, IFN-γ and IL-8. *Campylobacter fetus* causes Bovine Genital Campylobacteriosis which is a reproductive problem in livestock characterized by infertility and abortions. In this study, expression of IL-1β, TNF-α, IL-8 and IFN-γ in bovine endometrial epithelial cells infected with *C. fetus* was evaluated. The results obtained showed that IL-1β and IL-8 mRNA peaked at few minutes after cell infection and they decreased over time. IFN-γ increased through time peaking at 4 h p.i. TNF-α didn't change during infection. Also, in order to determine if *C. fetus* invasion was necessary for induction of these cytokines, we performed a cytoskeleton inhibition assay. IL-1β and IL-8 were downregulated when invasion was avoided. The present study examined the proinflammatory response to *C. fetus* in bovine endometrial epithelial cells. The bovine endometrial epithelial cells were able to recognize *C. fetus* resulting in an early proinflammatory response. Additionally, the internalization of bacteria is necessary to induce IL-1β expression, suggesting the importance of intracellular *C. fetus* recognition.

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) Leucinerich repeat-containing receptors (NLRs), C-Type Lectin-Like Receptors and, Cytoplasmic Nucleic Acid Sensors (Bryant *et al.*, 2015). This sensing enables innate immune system identify the source of infection and determine the type of immune response. Innate immune response includes proinflammatory cytokines secretion which recruits and activates immune cells to eliminate the pathogenic microorganisms (Iwasaki and Medzhitov, 2015).

The mucosal surface of the female reproductive tract, in contrast with other surfaces, interacts with sexually transmitted pathogens, allogeneic spermatozoa and fetus. It forms a physical and immunological barrier. These cells recognize pathogens and stimulate the underlying immune cells, producing an inflammatory reaction via production of cytokines resulting in adaptive immunity activation. They also produce antimicrobial peptides that eliminate several bacterial and viral agents (Aflatoonian *et al.*, 2014).

Some pathogens are able to evade this immune response. Keo et al., (2011) described the *Campylobacter jejuni* polysaccharide capsule, which confers human serum and cationic antimicrobials resistance. This capsule is necessary for mouse colonization and decrement in IL-17 secretion (Maue *et al.*, 2013). Stephenson et al. (2013) described a lipid A modification in *C. jejuni*, that confers bacteria the ability to avoid human TLR4 recognition. This immune evasion blocks the activation of immune response in mouse model enabling colonization (Vallance *et al.*, 2014). Day, Semchenko and Korolik, (2012) reported that *C. jejuni* glycoconjugates, necessary for host adhesion, mimic human gangliosides protecting the infective agent from the host's immune response through molecular mimicry. De Zoete *et al.*, (2010) found that *C. jejuni* flagellum evades TLR5 recognition through aminoacid sequence variation in a conserved region. This region is necessary to bind and activate TLR5 (Song *et al.*, 2017).

Campylobacter fetus causes Bovine Genital Campylobacteriosis (BGC), a reproductive disease of cattle associated to infertility and sporadic abortions which represents a serious problem in animal production (Mshelia *et al.*, 2010). Heifers infected with *C. fetus* showed minimal inflammatory reaction with few mononuclear and polymorphonuclear cells in vagina and cervix, and a moderate endometritis and salpingitís (Cipolla *et al.*, 1994). This minimal inflammation could be explained by external membrane composition, *C. fetus* possess lipooligosaccharides (LOS), instead of lipopolysaccharide (LPS) (Preston and Penner, 1987; Moran, Penner and Aspinall, 2002). *C. fetus* has a protein layer known as S-layer (surface layer). This layer protects against complement opsonization-phagocytosis response and avoid recognition by host innate immune system receptors (Blaser *et al.*, 1987; Fogg *et al.*, 1990; Blaser, 1993; Wormser and Angulo, 2009). Inflammatory response against *C. fetus* has not been previously described, in this study bovine endometrial epithelial cells were infected with *C. fetus* and cytokine patterns induced was analyzed. The results show that C. fetus induces proinflammatory citokines at early infection stages, and that invasion of this bacterium to this type of cells is necessary for cytokines induction.

METHODOLOGY

Bacterial strains and culture conditions.

Campylobacter fetus ATCC 27374 (Lage *et al.*, 2017) was grown at 37°C for 48 h under microaerophilic conditions on *Campylobacter* selective agar supplemented with 5% sheep blood. *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 (Prokaryotes 2005) was grown on hyperosmolar Luria Bertani broth at 37 °C for 12 h.

Epithelial cells isolation from endometrium.

Epithelial cells from endometrium were recovered using Skarzynski et al. (2005) protocol with some modifications. Uterus was removed from three sacrificed cows 15 min after exsanguination, tissue was washed with Hank's solution supplemented with 1.6 mg/ml of gentamicin and transported to the laboratory in the same solution on ice. The endometrium was cut in little pieces and washed three times with phosphate buffered saline solution (PBS) pH 7.2 (NaH_2PO_4 1.9 mM, Na_2HPO_4 8.1 mM, NaCl 154 mM), 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 –DMEM- supplemented with 10% fetal bovine) serum was added and incubated at 37 °C in oscillation for 2 h. The supernant was recovered and centrifuged at 4000 x g for 10 min. The pellet obtained was washed three times with DMEM:PBS (1:1). The pellet was resuspended in 5 ml of DMEM supplemented with 10% fetal bovine serum and filtered with a 40 µm strainer. Cells were placed on cell culture dishes, Hepes (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) 25 mM and antibiotic/antifungal (penicillin G 10,000 U, streptomycin 5,000 µg, and amphotericin B 12.5µg) were added. For fibroblast depuration, one minute tripsinization were performed every day for three days. Cell type was confirmed by RT-PCR (Campos-Múzquiz *et al.*, 2019). Also, an immunofluorescence was performed in order to observe cytokeratin 18 (Santa Cruz Biotechnology).

Invasion assays

C. fetus was grown on *Campylobacter* selective agar supplemented with 5% sheep blood for 48 h at 37 °C in microaerobiosis. Bacteria were diluted in DMEM to 10 X10^8 CFU/ml determined by spectrophotometry and plating. Epithelial cells of endometrium were seeded at 200,000 cells per 25 cm^2 culture flask. After washing three times with PBS, epithelial cells were infected with C. fetus with a multiplicity of infection (MOI) of 1000:1. Flasks were centrifuged at 165 x g for 3 min to maximize bacteria-cell contact and incubated for 2 h at 37 °C. Following the incubation, the monolayers were washed three times with PBS and incubated with DMEM supplemented with 10% fetal bovine serum and Hepes 25mM, and 30µg/mL gentamicin at 37 °C. Medium was recovered at 0, 2 and 4 h post infection. The cells from one repetition were lysed with triton X100 1% and 50 µL of each well were plated 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, catalog number CN0025A, England). The rest of the cells of the assay were treated with TRIzol in order to recover eukaryotic and prokaryotic RNA. Three independent assays with three replications of each time were performed. For intracellular surviving, we also performed a quantitative RT-PCR **(**Power SYBR green **–**Fermentas**-**) as describe by Fey *et al.*, (2004). A standard curve was built with 1 ng, 100 pg, 10 pg, 1 pg y 100 fg RNA of *Campylobacter fetus* ATCC 27374 or *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028. Primers used were 5'-GGCAATATCATAGAAAATCCGTTATC -3' and 5'-TCCTGCTCTTTCATTTGCTT -3' for *C. fetus,* these primers amplified a 161pb fragment from fumarate reductase gene (*frdA*). For *Salmonella* quantitation *rpoD* primers were used (Botteldoorn *et al.*, 2006). Specificity of the PCR product was confirmed by analysis of the dissociation curve. Only standard curves with an R^2 above 0.96 were considered for quantification.

Cytoskeleton inhibition assays

A 100 mM cytocalasin D (Sigma-Aldrich) stock was prepared in dimethyl sulfoxide. Gentamicin protection assays were performed as described above, with some modifications. 200, 000 cells were seeded in a 25 ml culture flask 12 h before assay. Then cells were washed three times with PBS, and DMEM with cytochalasin D (3 mM) was added to the flasks. Host cells were incubated for 30 min at 37 °C. Then invasion assays were performed with *Campylobacter fetus* (MOI 1:1000) and with *Salmonella* (MOI 50:1). Inoculum and inhibitors were removed, and cells were washed three times with PBS. Then DMEM supplemented with 10% fetal bovine serum and Hepes 25 mM, and 30 µg/mL gentamicin was added. The cells from one repetition were lysed with triton X100 1% and 50 µL of each well were plated 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, catalog number CN0025A, England). The rest of the cells of the assay were treated with TRIzol in order to recover eukaryotic and prokaryotic RNA. Three independent assays with three replications of each time were performed.

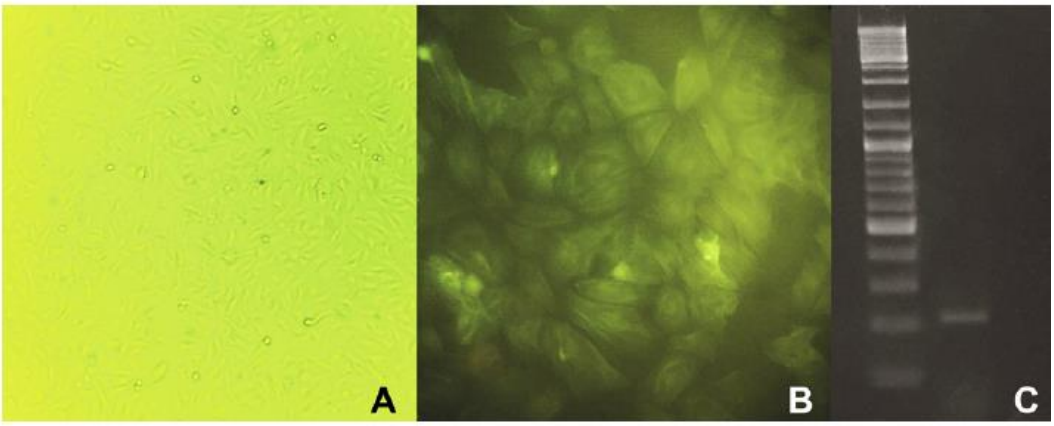
Measuring of proinflammatory cytokines on infected cultures

RNA was extracted following TRIzol protocol. cDNA was synthetized using ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs) following its protocol. We used Oligo dT primers for eukaryote mRNA and random primers for prokaryote mRNA. A quantitative PCR (Maxima SYBR green -Thermo Fisher-) was performed using primers: TAF2 (5'-CATCTCCTGGAACCCAGAAA-3', 5'-GGCTGTTCTCCTCAATCTGC-3', 98 pb), B-actin (5'-AAATCGTGCGTGACATTAAG-3', 5'-GAGTACTTGCGCTCAGGAG-3', 341 pb) and GPDH (5'-GCCATCACCATCTTCCAGG-3', 5'-GGTAGTGGAGACCCCAGTGG-3', 115pb), as reference genes; and IL-1β (5'-GAAAGAGACAACAAGATTCCTGTGG-3', 5'-GGTCTACTTCCTCCAGCTGCA-3', 108 pb), TNF-α (5'-CATCTACTCRCAGGTCCTCTT-3', 5'-GCAATGCGGCTGATGGT-3', 82 pb), IL-8 (5'-AGTACAGAACTTCGATGCCAATG-3', 5'-GTAAGCTTAACAATTTCTGAATTTTC-3', 127pb), IFN-γ (5'-GGGTTTTTCTGGTTCTTATGGC-3', 5'-GTCACTTTCATCTTTCCAATTCTT-3', 144 pb) as ????. Specificity of the PCR product was confirmed by analysis of the dissociation curve. The amplification efficiency (E) of each gene was calculated from the standard curves using the equation $E = ((10^{-1 / slope})-1)*100$. The value of the slope when cDNA dilution was compared to ΔCt (Livak and Schmittgen, 2001), so we used the comparative Ct method (2-ΔΔCt) (Schmittgen and Livak, 2008). For gene normalization we obtained geometric average of the three reference genes (Vandesompele *et al.*, 2002). Analyses of cytokine expression were realized with treated cells with cytochalasin D for 30 min in order to determine if the inhibitors could induce cytokine expression.

Statistical analysis

For determining differences in RNA copies and CFUs through time, an F test was performed to check the equality of the variances of the two samples. All variances were equal, so a two sample T test was performed comparing time 2 h p.i and 4 h p.i against 0 h p.i. These analyses were performed using R. Delta Ct was obtained from each gene with the geometric average of the reference genes, an F test was performed to check the equality of the variances of the two samples and Student T test was performed comparing ΔCt of treatment versus ΔCt of control.

RESULTS.



Primary endometrial epithelial cells cultures were established and confirmed by RT-PCR and immunofluorescence (Figure 1).

Figure 1. Epithelial cells from bovine endometrium maintained in DMEM supplemented with 10% fetal bovine serum. A) Normal appearance of the epithelial-like cells (20×). B) Immunofluorescence. Cytokeratin 18 of epithelial cells of bovine endometrium stained with Alexa 488 (green) (40×). C) The results of RT-PCR for keratin 8 on 2% agarose gel after staining with ethidium bromide. In left lane: DNA ladder (Thermo Fisher Scientific); right lane: the amplicon of keratin 8 (215 pb).

Invasion assays.

Intracellular *C. fetus* decreased over time, indicating a reduction of intracellular bacteria (p=2.2e-16). At 0 h post-infection (p.i.) the average (~)

colony-forming units (CFU) were 22,408 per flask (CFU/flask). 2 h p.i. that number decreased to ~1,316 CFU/flask and at 4 h p.i. there were ~233 CFU/flask (Figure 2). Control cells infected with *Salmonella*, the CFU increased through time indicating intracellular replication of bacteria (p = 1.49e-10). At 0 h p.i. the average of intracellular bacteria were ~42,150 CFU/ flask, at 2 h p.i. the CFU decrease to ~37,125 CFU/ flask, and at 4 h p.i. they proliferated and increased to 72,925 CFU/flask.

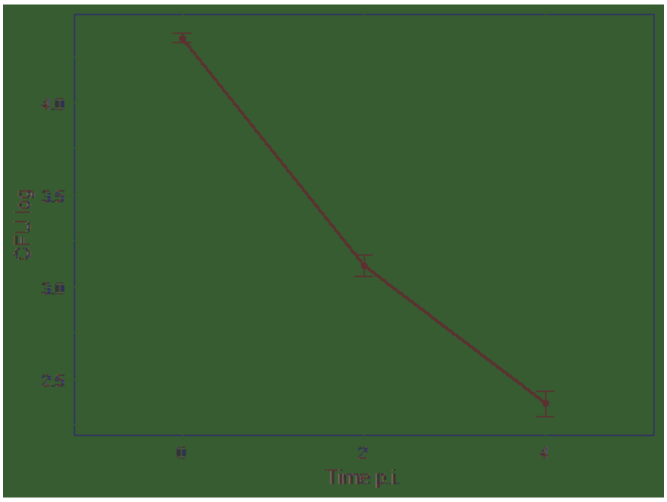


Figure 2. CFU of *C. fetus* plated on blood agar. A gentamicin protection assay was performed in order to demonstrate *C. fetus* was invading bovine endometrial epithelial cells.

In order to confirm these results, mRNA quantification assays was performed. For *C. fetus frdA* analysis (constitutive gene), showed that mRNA copies (RC) decreased significantly during invasion assays (p = 0.002). At 0 h p.i. the number of RC were ~238,107 RC/flask, at 2 h p.i. they decreased to ~74,552 RC/ flask, and at 4 h p.i. there were 31,063 RC/flask (Figure 3a). For *Salmonella* quantification of *rpoD* showed that RC of this constitutive gene increased through time (p = 1.586e-06). At 0 h p.i. there were ~112,890 RC/flask, then they increased to 573,027 RC/flask and they keep increasing up to 8,669,563 RC/well (Figure 3b).

A

B

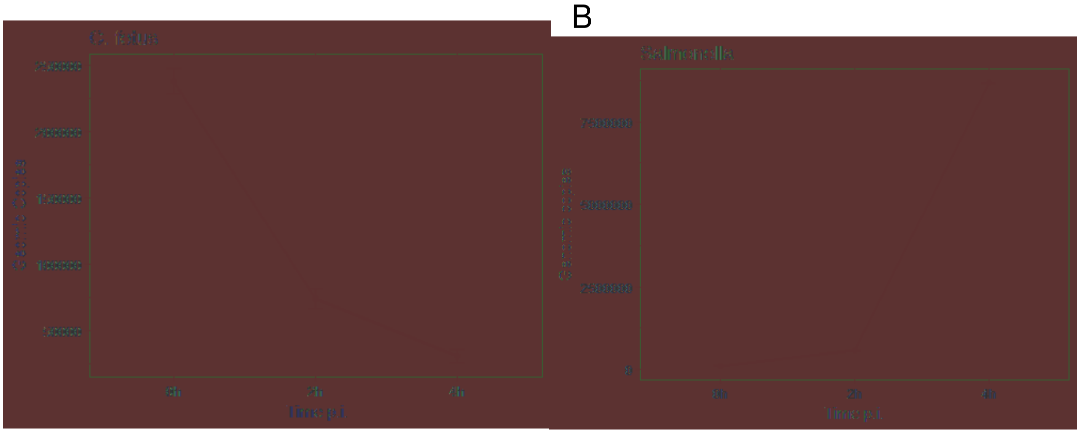


Figure 3. Intracellular survival of *C. fetus* and *Salmonella* in bovine endometrial epithelial cells. An invasion assay was performed and RNA was extracted from cells. cDNA was synthetized using random primers, and a quantitative PCR was performed. A) Cells infected with *C. fetus* and B) cells infected with *Salmonella*.

Cytokine expression analysis.

Bovine endometrial epithelial cells invaded by *C. fetus* or *Salmonella* expressed IL-1β, IL-8, and IFN-γ, demonstrating *C. fetus* can be sensed by these cells, nonetheless cells didn't changed TNF-α expression (Figure 4).

C. fetus-invaded cells showed their maximum IL-1β expression (4.65 fold change) early (0 h p.i.) suggesting an acute response. *Salmonella*-invaded cells showed maximum IL-1β expression (3.56 fold change) at 4 h p.i. When comparing IL-1β expression in *C. fetus* invaded cells and *Salmonella* invaded cells at these times, it can be noted that *C. fetus* invaded cells expressed more IL-1β, with a difference of 1.09 fold change (p =6.645e-05). Also, they showed a different pattern of expression. In *C. fetus* invaded cells the expression of IL-1β decreased over time (p = 7.492e-05). At 0h p.i. there was an initial fold change of 4.65, and it decreased to 3.78 fold change at 2 h p.i and continued this tendency until at 4 h p.i. the expression was 1.09 fold change. On the other side, when invaded with *Salmonella*, cells expressed IL-1β in an increasing way (p = 0.0007) from time 0 h (0.84 fold change) to 4 h p.i. (3.56 fold change) (Figure 4).

The pattern of expression of IL-8 was the same when infected with *Salmonella* as with *Campylobacter*. In both cases, IL-8 was expressed with higher fold change at 0 h p.i, 3.41 when exposed to *C. fetus* and 6.14 when exposed to *Salmonella*, and it decreased through time (p = 0.031, p = 5.127e-05 respectively) at 4 h p.i., when the fold change of *C. fetus*-invaded cells was 2.28 and 2.68 when exposed to *Salmonella*. There was no difference in the expression between both treatments (p-value = 0.4401) (Figure 4), suggesting endometrial epithelial cells induce polymorphonuclear cells infiltration with the same intensity when *C. fetus* or *Salmonella* are present.

IFN-γ behaved different than the previous cytokines described. When infected with *Salmonella*, the epithelial cells didn't change the expression of IFN-γ. At 0 h there was a fold change of 0.5, at 2 h p.i. 0.74 and at 4 h p.i the fold change was 0.85 but this difference was non-significant ($p = 0.367$). However, when exposed to *Campylobacter*, its fold change increased through time significantly ($p = 0.002$). At 0 h p.i. the fold change was 0.89, at 2 h p.i it increased to 2.77 and at 4 h p.i it reached 3.62 fold change (Figure 3), suggesting *C. fetus* can´t modulate host immune response. TNF-α expression didn't change compared to control in any treatment at any time .p.i.

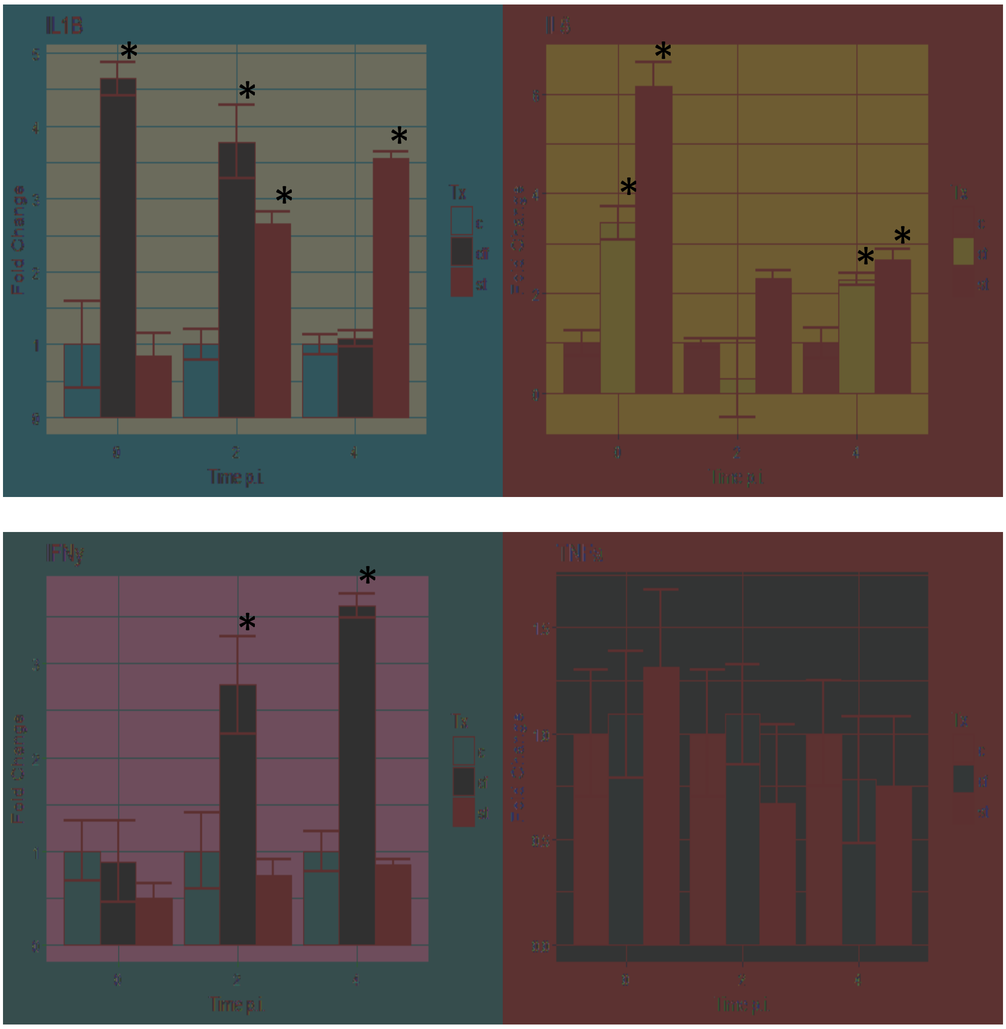


Figure 4. Interleukin expression in bovine endometrial epithelial cells challenged with *C. fetus* or *Salmonella*. Expression was analyzed with a 2 delta CT and compared to control cells (no infected). A student T-test was performed to delta Cts (Ct gene of interest – geometric media Ct housekeeping genes) compared against non-treated cells. *p<0.05.

Then, in order to determine if *C. fetus* invasion was necessary for induction of these cytokines, we performed a cytoskeleton inhibition assay so *C. fetus* couldn´t be internalized in epithelial cells and performed a relative quantification of IL-1β, IL-8, IFN-γ and TNF-α.

When *C. fetus* invasion was inhibited, IL-1β expression was decreased with a fold change of 0.34 ($p = 0.005$), meaning it is necessary for *C. fetus* internalization for the epithelial inflammatory response. A similar trend was observed with IL-8, nonetheless, the difference was non-significant with a fold change of 0.53 ($p = 0.105$). TNF-α presented a fold change of 0.95 which was non-significant either ($p = 0.1375$). However, IFN-γ was increased to a 1.90 fold change ($p = 0.021$), possibly because of cytochalasin D toxic effect (Figure 5).

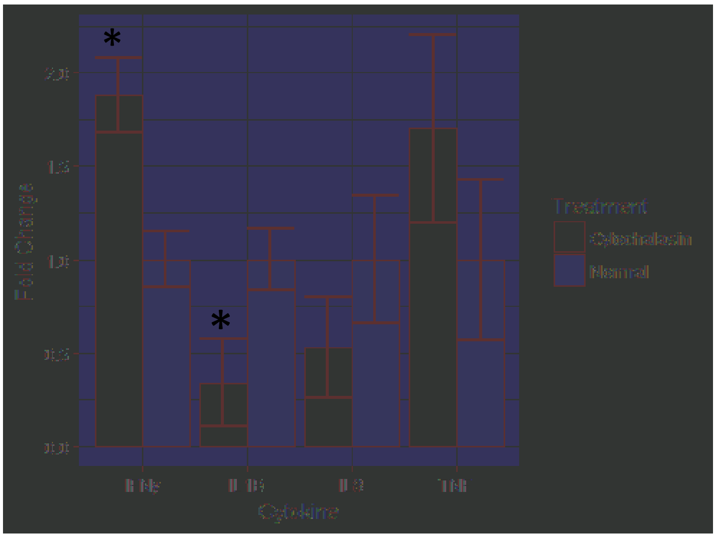


Figure 5. Interleukin expression in bovine endometrial epithelial cells treated with cytochalasin D and challenged with *C. fetus.* Expression was analyzed with a 2 delta CT and compared to control cells (non cytochalasin D-treated cells). A student T-test was performed to the delta Cts (Ct gene of interest – geometric media Ct housekeeping genes) comparing against non cytochalasin D-treated cells. *p<0.05.

DISCUSION

The PRRs are responsible for sensing the presence of pathogens by recognizing conserved structures among microbial species, called pathogen-associated molecular patterns (PAMPs). The sensing of PAMPs upregulate the transcription of proinflammatory cytokines genes like IL-1β, TNF-α and IL8 (Takeuchi and Akira, 2010). In this study we demonstrate that *Campylobacter* *fetus* is recognized by endometrial epithelial cells. A RT-qPCR performed after an intracellular survival assay showed that *C. fetus* induced the expression of proinflammatory cytokines in bovine endometrial epithelial cells. These results agreed with previous studies using other *Campylobacter* species. Arce *et al.*, (2010) showed that *Campylobacter rectus* was able to induce proinflammatory cytokines expression on trophoblast epithelial cells; as well as Day *et al.*, (2010) demonstrated that Caco-2 cells challenged with *Campylobacter concisus*, *Campylobacter showae* and *Campyloacter hominis* secreted proinflammatory cytokines.

Also, we showed that *C. fetus* induced IL-1β expression in endometrial epithelial cells at 0 h p.i. indicating an early induction of proinflammatory cytokines. This same phenomenon was observed in HEp-2 and HT-29 cells infected with *C. jejuni*, where after 2 h of infection IL-1β was detected by ELISA (Yu, Nanthakumar and Newburg, 2016). Also, *Campylobacter rectus* promote IL-1β expression 2 h p.i. in HEp-2 cells (Wang, Kraig and Kolodrubetz, 2000).

The IL-1B expression in epithelial cells infected with *C. fetus* was earlier than *Salmonella* (0h p.i vs 4h p.i respectively) and it decreased through time. In *C. fetus* genome there are not virulence factors identified besides S- layer (Kienesberger *et al.*, 2014), therefore induction of cytokines could be faster than *Salmonella,* resulting in bacteria elimination. These results agree with the pattern of expression observed previously in 4-week old chicken challenged with S. Typhimurium or *C. 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). The decrease of IL-1β could be due to IL-10 increase (Murthy *et al.*, 2000). Stephenson *et al.*, (2014) showed that *C. jejuni* flagellum binds to Siglec-10 of dendritic cells and resulted in increased IL-10 expression. Nonetheless, more studies need to be performed. On the other hand, we observed that when cells are infected with *Salmonella*, pro-inflammatory cytokines expression takes longer than *C. fetus* to reach the peak of expression. This could be due to *Salmonella* adaptation to intracellular niche (Larock, Chaudhary and Miller, 2015). For example, *Salmonella* uses SpvD, an effector protein secreted through type 3 secretion system, in order to avoid nuclear translocation of NF-kB transcriptional factor. Thus, infected cells reduce pro-inflammatory cytokines secretion (Rolhion *et al.*, 2016). Furthermore, when *C. fetus* internalization was inhibited, IL-1β expression was halved, demonstrating that *C. fetus* is also recognized intracellularly and that this recognition is required to induce higher inflammation.

The pattern of expression of IL-8 was similar to IL-1β, suggesting that *C. fetus* infection promotes an early neutrophil recruitment (Foley *et al.*, 2012). The decrease of IL-8 could be also due to IL-10 increase as described with IL-1 β (Méndez-Samperio *et al.*, 2002) or due to IFN-γ upregulation since it represses genes involved in leukocyte recruitment (Hoeksema *et al.*, 2015). Moreover, when *C. fetus* internalization was inhibited, the expression of IL-8 was not altered, indicating that the external sensing and not the internalization of the bacteria induce polymorphonuclear recruitment (Takeuchi and Akira, 2010). This is in agreement with the study presented by (Tajima *et al.*, 2007) in which non-internalized commensal *Staphylococcus aureus* induced IL-8 just as the internalized commensal bacteria. Also, IL-8 expression of *Salmonella*- infected cells agreed with a work previously described (Arce *et al.*, 2010) in which IL-8 expression of IPEC-J2 cells increased from 2 h p.i. after *Salmonella* LPS exposure.

IFN-γ increased over time presenting an inverse expression pattern of that observed in IL-1β and IL-8. These results are consistent with the observed in INT-407 cells infected with *C. jejuni* in which IFN-γ peaked at the 12 hours post stimulation. IFN-γ alters epithelial barrier function during inflammation by disrupting tight cell junctions and increasing permeability in polarized epithelial cells, leading to cytosolic translocation of occludins, claudins, and JAM-A (Bruewer, 2005). The increment of IFN-γ expression would help *C. fetus* translocate to another tissue compartment as with *C. jejuni*. In the presence of both, IFN-γ and *C. jejuni,* a synergistic effect is shown whereby bacterial translocation across the monolayer is significantly greater than in absence of IFN-γ. Also, this IFN-γ expression suggests that epithelial endometrial cells could be involved in M1 macrophages activation, and in adaptive immunity modulation, like antigen presentation and TH1 response to campylobacteriosis (Hoeksema *et al.*, 2015). On the other hand, INF_{γ} increased when cells were treated with cytochalasin D, this could be due to a synergistic effect of *C. fetus* sensing and the toxic effect of cytochalasin D. It has been reported before that cytochalasin D activates p53 transcriptional factor which besides controlling DNA damage stress signaling, it also controls apoptosis and hypoxia, thus, gene expression is modified (Rubtsova *et al.*, 1998).The expression of IFN-γ in *Salmonella* infected cells was non-significant. These results are not surprising since *Salmonella* possess an immune response modulator IpaJ which decreases IFN-γ expression in macrophage cell line HD11 (Yin *et al.*, 2018). Also, the principal source of IFN-γ during *Samonella* infection are the neutrophils and NK cells (Pham and McSorley, 2015)).

When cells were infected with *Salmonella* or invaded with *C. fetus* we didn´t observe a change in TNF-α expression. The same phenomenon occurred when Cronin *et al.*, (2012) challenged bovine endometrial epithelial cells with lipopolysaccharide, they couldn't detect TNF-α in cell supernatant. These same results were observed in bovine endometrial epithelial cells challenged with danger associated molecular patterns (DAMPs) (Healy, Cronin and Sheldon, 2014). Possibly, TNF-α expression didn't change because its expression is subjected to different conditions since the main function of the endometrium is blastocyst implantation (Kaneko, Day and Murphy, 2013).

In conclusion, the present study examined the proinflammatory response to *C. fetus* in bovine endometrial epithelial cells. The bovine endometrial epithelial cells were able to recognize *C. fetus* resulting in an early proinflammatory response. Additionally, the internalization of bacteria is necessary to induce IL-1β expression but not IL-8, suggesting the importance of intracellular *C. fetus* recognition. The incapacity to survive inside cells, the early induction of cytokines and the upregulation of IFN-γ suggests that the aim of *C. fetus* in uterine cavity is to cross tight junctions of epithelium, just like it was demonstrated in human cells HT-29/B6 and Caco-2 cells (Baker and Graham, 2010; Bücker *et al.*, 2017).

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DISCUSIÓN GENERAL

En este estudio se establece que *Campylobacter fetus* tiene la capacidad de adherirse e invadir células epiteliales de endometrio bovino. Además, se describe la respuesta inmunitaria que se induce en las células epiteliales de endometrio bovino al entrar en contacto con este microorganismo.

En este estudio se estableció un modelo de infección *in vitro* para *C. fetus,* con el cual se mostró que el microorganismo puede adherirse e invadir células epiteliales de endometrio bovino. Anteriormente se había demostrado la capacidad de *C. fetus,* para adherirse a células Hep-2 (Graham and MacDonald, 1998) y a células MDBK (Chiapparrone *et al*., 2014); así como su capacidad para invadir células Caco-2 (Baker and Graham, 2010). Sin embargo, ya que estas células son de origen humano, era necesario confirmar que esto sucediera en el hospedador final de *C. fetus*, el bovino. En el caso de las células MDBK, aunque son de origen bovino, éstas provienen de riñón, el cual no es el órgano hacia el cual *C. fetus* tiene tropismo. Así mismo, en ese modelo de infección, sólo demostraron que las bacterias tenían la capacidad para adherirse pero no mencionan algo acerca de la invasión a las células.

Aún se desconoce el mecanismo por el cual *C. fetus* entra a las células epiteliales de endometrio bovino. En cambio, en otras especies de *Campylobacter* se han descrito los mecanismos por los cuales invaden a las células. Por ejemplo, *C. jejuni* expresa las proteínas de unión a fibronectina CadF y FlpA durante la invasión de células epiteliales (Monteville, Yoon and Konkel, 2003; Larson *et al*., 2013; Patrone *et al*., 2013). Asimismo Eucker (2012) demostró que estas proteínas de unión a fibronectina inducen su internalización en células INT407 mediante las adhesiones focales ya que cuando CadF y FlpA se unen a la fibronectina, el receptor del factor de crecimiento epidermal y las adhesiones focales se activan, llevando a la formación de extensiones de la membrana. No obstante, *C. fetus* posee en su genoma la proteína CadF (GenBank: AIR78335.1), por lo que posiblemente esta pudiera ser la forma en que se internaliza a las células, sin embargo aún no se ha demostrado su expresión.

Por otra parte, Watson y Galán (2008) demostraron que *C. jejuni* utiliza los microtúbulos para internalizarse. En el caso de *Campylobacter upsaliensis*, se ha demostrado que la bacteria utiliza microtúbulos y actina para su internalización en células Caco-2 (Mooney *et al*., 2003). En las formas de invasión que se han descrito *C. jejuni* las proteínas importantes para ésta son las caveolinas y las integrinas. En el 2008, Watson y Galán reportaron que en células T84 tratadas con metil-beta ciclodextrina, un agente secuestrador de colesterol, se bloqueaba la internalización de *C. jejuni*. También, al exponer a una mutante de caveolina-1 la internalización de *C. jejuni* disminuyó significativamente y no permitieron su unión con el factor de crecimiento epidermal (EFG), el cual forma parte de las adhesiones focales, y por lo tanto *C. jejuni* no pudo internalizarse (Konkel *et al*., 2013). Tres años después, Konkel y colaboradores (Konkel *et al.*, 2013) demostraron que esta invasión era dependiente de la presencia del colesterol pero independiente de caveolas. En cuanto a la utilización de estructuras del citoesqueleto, *C. fetus* difiere de estas dos especies (*C. jejuni* y *C. upsaliensis)*, ya que la inhibición de la polimerización de los microtúbulos no afectó su capacidad de invasión en este estudio.

Además, en este estudio se muestra a través de un ensayo de sobrevivencia a gentamicina y por la cuantificación de mRNA del gen *frdA* que *C. fetus* no se replica dentro de las células epiteliales. Al momento de sembrarla después de lisar con un detergente a las células invadidas por *C. fetus*, se obtuvieron cantidades menores de unidades formadoras de colonias a través del tiempo. También la cuantificación del mRNA de *frdA* se comportó de la misma manera. Estos dos hechos sugieren tres situaciones: primero la posibilidad de que la bacteria se esté eliminando al unirse el endosoma con el lisosoma; segundo, que la bacteria haya entrado en un estado viable no cultivable; o tercera, que la bacteria esté haciendo transcitosis.

La primera situación pudiera ser cierta ya que hasta el momento no se han identificado factores de virulencia que le permitan la evasión de la unión endosoma-lisosoma (Kienesberger, 2014). Los únicos factores de virulencia que se ha descrito hasta el momento son la capa S (Fogg *et al.*, 1990; Blaser, 1993), una toxina citoletal (Asakura *et al.*, 2007) y el sistema de secreción tipo 4 VirB/VirD4 el cual sirve para translocar ADN y es necesario para la invasividad de células epiteliales *in vitro* (Gorkiewicz *et al.*, 2010). También posee un flagelo que pudiera actuar como factor de virulencia, ya que en *C. jejuni* sirve para translocar las proteínas Cia al citoplasma las cuales son necesarias para la invasión (Konkel *et al.*, 2004; Neal-McKinney and Konkel, 2012) aunque hasta el momento no se han identificado genes que codifiquen para este tipo de proteínas en *C. fetus*.

En el segundo caso, el estado de forma viable no cultivable se ha identificado en la bacteria filogenéticamente cercana, *Campylobacter jejuni*, al invadir células epiteliales de intestino Caco-2 (Watson y Galán, 2008). Hasta el momento no se ha realizado un análisis del transcriptoma de *C. jejuni* en este estado, por lo que aún se desconoce si los genes expresados en este estado están presentes en el genoma de *C. fetus*, pero cabe la posibilidad, pues presentan el 88% de similitud (Piccirillo *et al.*, 2016).

El estado de viable no cultivable es un estado de dormancia en el cual algunas bacterias tienen la capacidad de entrar cuando se enfrentan a condiciones ambientales adversas, como cambios de temperaturas, cambios de pH (Besnard *et al.*, 2002), inanición (Cook and Bolster, 2007), y disponibilidad de oxígeno (Kana *et al.*, 2007). Las bacterias en este estado no son cultivables pero son viables, lo que significa que son metabólicamente activas. Sin embargo, aún es necesario demostrar que este es el caso de *C. fetus*.

En el tercer caso, al parecer *C. fetus* tiene la capacidad de translocar células epiteliales Caco-2 polarizadas sin alterar la resistencia eléctrica transepitelial (Baker y Graham, 2010), lo que sugiere que en vez de atravesar la monocapa entre las células, está atravesando las células, pudiendo presentarse este fenómeno en las células epiteliales del endometrio bovino. Sin embargo, Bucker et al., (2017) menciona que cuando *C. fetus* se transloca a través de una monocapa de células HT-29/B6 se alteran las uniones estrechas de ésta, sugiriendo el paso intercelular de la bacteria. No obstante aún es necesario probar el fenómeno células polarizadas de epitelio bovino.

El hecho que *C. fetus* no se replique dentro de las células epiteliales no significa que no tenga la capacidad de causar infección en otro tejido y por lo tanto iniciar una respuesta inmunitaria para su eliminación. La respuesta inmunitaria está mediada por células de la respuesta inmune, así como por células epiteliales (Abreu, 2010; Montazeri *et al.*, 2015). Las células epiteliales tienen como función crear una barrera impermeable que evite el paso de patógenos hacia las capas más profundas y secretar substancias microbicidas (Fahey, 2005; Kaneko, Day and Murphy, 2013). Además de iniciar una respuesta contra patógenos, el epitelio del endometrio bovino tiene que discernir entre un patógeno y un feto semialógeno, para modular la respuesta inmune y evitar abortos.

Anteriormente, debido a estudios histopatológicos se había sugerido que la respuesta inflamatoria inducida por *C. fetus* era menor comparada con la de otras bacterias patógenas aisladas del útero bovino. *C. fetus* induce una inflamación ligera con poca infiltración de linfocitos y neutrófilos en cérvix y vagina. También puede producir una endometritis difusa subaguda a crónica superficial (Cipolla *et al.*, 1994). Los cambios principales que se presentan en el endometrio con vibriosis son la degeneración e hiperplasia ligera en el epitelio superficial e infiltración de linfocitos y netrófilos moderada en el estroma. Las lesiones se pueden extender hasta 120 días. No se presenta piometra (Estes, Bryner and O'Berry, 1965). Al contrario de *C. fetus*, *Escherichia coli,* la bacteria más comúnmente aislada de úteros postpartos infectados, induce discontinuidad en el epitelio endometrial, desprendimiento de éste, infiltración exacerbada de polimorfonucleares al lumen uterino, hiperplasia estromal y degeneración glandular (Williams *et al.*, 2008; Dar *et al.*, 2016).

En este estudio se demuestra que *C. fetus* induce expresión de citocinas proinflamatorias, y que ésta se ve disminuida rápidamente durante la infección, lo cual podría explicar el tipo de endometritis que se ha descrito en la vibriosis bovina (Cipolla *et al.*, 1994).

La respuesta inmunitaria de las células endometriales se ve modulada por la presencia de algunas hormonas implicadas en la reproducción, principalmente el estradiol. El estradiol aumenta la secreción del antimicrobiano SLP1 y disminuye la respuesta de los TLR en células ECC-1 (Fahey, 2005). Los receptores de estradiol, una vez activados por el estradiol, son translocados al núcleo y modulan de forma epigenética la expresión de algunos genes inmunitarios. ERα y el factor transcripcional NF-κB p50-p65 interactúan forman un complejo que recluta la histona deacetilasa HDAC1 y la demetilasa JARID1b las cuales disminuyen la metilación y acetilación de las histonas río arriba del gen del TLR9. También el estradiol promueve la síntesis de IκBα, de esta forma inhibe la translocación del NF-κB al núcleo y por lo tanto se inhibe la expresión de todas las citocinas que promueve este factor transcripcional (Kovats, 2015). Ya que *C. fetus* se transmite durante la monta, esto significa que la vaca se encuentra en la fase del estro, por lo tanto los niveles de estrógeno se encuentran altos y la respuesta de las células epiteliales ante *C. fetus* no es la óptima para eliminarla.

Las endometritis causan grandes pérdidas económicas. Debido a la infertilidad consecuente a ésta, la tasa de concepción se disminuye hasta un 50% y los días abiertos aumentan al menos 30 días, por lo que la producción de leche en el hato se disminuye, aumentando el número de cabezas sacrificadas por fallas reproductivas. Se calcula que un solo caso de endometritis cuesta casi 300 euros y anualmente E.U.A pierde 650 millones de dólares a causa de la endometritis (Williams *et al.*, 2008). Sin embargo este es sólo el costo de las endometritis agudas postparto. La campilobacteriosis, por el contrario, induce una infección subclínica que sólo se observa como una falla reproductiva en la mayoría de los casos, aumentando el número de sacrificio de estos animales.

En México, se considera a la vibriosis genital bovina una enfermedad enzoótica. Se encuentra clasificada en el Diario Oficial de la Federación dentro del grupo 3 del “Acuerdo mediante el cual se dan a conocer en los Estados Unidos Mexicanos las enfermedades y plagas exóticas y endémicas de notificación obligatoria de los animales terrestres y acuáticos”. Esto significa que el riesgo es menor desde el punto de vista epidemiológico, económico, de salud pública y de comercio nacional e internacional, por lo que no existe un registro epidemiológico sobre el estatus actual del país en cuanto a esta enfermedad ya que no se realizan los reportes de ésta, aunque se encuentre en la lista de enfermedades de reporte obligatorio de la OIE 2018.

Uno de los métodos de prevención sugeridos por la OIE en el capítulo 2.4.4 “CAMPILOBACTERIOSIS GENITAL BOVINA” en el Manual de las Pruebas de Diagnóstico y de las Vacunas para los Animales Terrestres 2017 es la utilización de bacterinas (OIE, 2017). Sin embargo estas vacunas no son tan eficientes para la inmunización contra esta bacteria, ya que la bacteria presenta variación antigénica durante la infección (Garcia *et al.*, 1995; Michi *et al.*, 2016).

Es por esto que es necesario conocer la patogénesis de la bacteria, así como la respuesta inmunitaria inducida durante la infección. De esta forma se podrán planificar estrategias de control y erradicación más eficaces disminuyendo las pérdidas económicas de los productores.

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