An air-liquid interface model to investigate the niche preference of *Campylobacter fetus*

by

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Content

List of abbreviations

ALI	Air-liquid interface
BA plates	Blood agar plates
BEGM	Bronchial Epithelial Cell Growth Medium
BGC	Bovine genital campylobacteriosis
BHI	Brain Heart Infusion
BTUB	Beta-tubulin
CAZymes	Carbohydrate-active enzymes
C. coli	*Campylobacter coli*
Cdt	Cytolethal distending toxin
C. fetus	*Campylobacter fetus*
Cff	*Campylobacter fetus subspecies fetus*
CFSE	Carboxyfluorescein succinimidyl ester
Cft	*Campylobacter fetus subspecies testudinum*
CFU	Colonies forming unit
Cfv	*Campylobacter fetus subspecies venerealis*
C. jejuni	*Campylobacter jejuni*
DAPI	4′,6-diamidino-2 phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPBS-/-	Phosphate-Buffered Saline without calcium and magnesium
E. coli	*Escherichia coli*
FBS	Fetal bovine serum
FISH	Fluorescent in situ hybridization
GH	Glycoside hydrolases
HBSS-/-	Hanks' Balanced Salt Solution without calcium and magnesium
HEPES	Hydroxyethylpiperazine Ethane Sulfonic Acid
HI	Heart Infusion
Log	Logarithmic
MALII	Maackia amurensis lectin II
MOI	Multiplicity of infection
OD_{600}	Optical density at 600 nm
O/N	Overnight
ORF	Open reading frames
PenStrep	Penicillin-Streptomycin
PFA	Paraformaldehyde
RT	Room temperature
Sap	Surface array protein
S-Layer	Surface layer
SNP	Single nucleotide polymorphism
TEER	Transepithelial electrical resistance

Abstract

Campylobacter fetus subspecies venerealis (Cfv) and C*ampylobacter fetus subspecies fetus* (Cff) are pathogens primarily associated with reproductive problems in cattle and sheep. Cfv shows host specificity and tissue tropism, they are restricted to the genital tract of cattle, while Cff has a broad host distribution and can colonize different types of tissues. The virulence mechanisms underlying infection of *Campylobacter fetus (C. fetus),* and its hosts are poorly understood. The niche preference of *C. fetus* subspecies can be explained by comparing their ability to adhere to and invade host-specific epithelial cells.

An air-liquid interface (ALI) model, which is a more physiologically relevant model than immortalized cell lines, was developed using primary bovine uterine epithelial to explore the niche preference of *C. fetus.* Due to the low viability of isolated bovine cervical cells, porcine cervical epithelial cells were used instead for infection assay to test different infection conditions. Brain Heart Infusion was identified as the most optimal medium for liquid culturing of *C. fetus*.

The infection assay demonstrated the ability of Cff to adhere to porcine cervical epithelial cells. The highest adherence of cff cells was observed on epithelial cells infected with a multiplicity of infection of 50 after 1-hour incubation in BHI. These results show that ALI culture is a suitable model for studying the adherence of *C. fetus.* Therefore, further optimizing ALI models using bovine and ovine uterine epithelial cells is important to study *C. fetus* niche preference by comparing their adherence and invasion abilities.

Plain language summary

Campylobacter fetus (*C. fetus*) is a species of bacteria with three subspecies, two of which are bacteria that affect livestock: *Campylobacter fetus subspecies venerealis* (Cfv) and *Campylobacter fetus subspecies fetus* (Cff). Cff can be found in the intestines and reproductive system of different animals, including cows, sheep and humans, leading to different diseases, such as abortion. In contrast, Cfv is only found in the reproductive system of cows and bulls, causing abortions in cows. The reason why these two *C. fetus* subspecies live in different environments is poorly understood. These different preferences for living environments could be explained by comparing the ability of the *C. fetus* subspecies to bind to and cross through the reproductive cells of cows.

In this study, we worked on developing a cell model using the cervical cells of cows that mimics the environment of the reproductive system to better understand how *C. fetus* infects its hosts. Cultures of cells that were isolated from the cow's cervix did not grow well; therefore, we had to use cervical cells isolated from pigs as an alternative for the infection experiments. We tested different conditions for growing *C. fetus* and found that a nutrient-rich medium worked best. We also found that the best conditions for testing the infection experiments are: using a concentration of *C. fetus* and letting them grow with the cervical cells for one hour. These results show that this cell model is a suitable model for studying the binding of *C. fetus* to host cells. Therefore, optimizing this model using reproductive cells from cows and sheep is important as it helps us get closer to understanding the preferences of *C. fetus* subspecies for different environments.

1. Introduction

1.1 *Campylobacter fetus*

Campylobacter is a genus of s-curved, gram-negative, microaerophilic bacteria and most of the species have a single polar flagellum at one or both ends of the cell(1). The first Campylobacter species discovered, *Campylobacter fetus* (*C. fetus*), was isolated from aborting ewes in 1913 by McFadyean and Stockman (2). *C. fetus* has three subspecies, two of which are mammal-associated pathogens: *Campylobacter fetus subspecies venerealis* (Cfv) and C*ampylobacter fetus subspecies fetus* (Cff). The third subspecies, *Campylobacter fetus subspecies testudinum* (Cft), is a reptile-associated pathogen and is genetically divergent from the other two subspecies (3). Therefore, Cft will be excluded from this study.

1.2 Pathogenicity and niche preference

Both Cff and Cfv are important pathogens affecting livestock. Cfv is the source of bovine genital campylobacteriosis (BGC), a sexually transmitted disease characterized by infertility, early embryonic mortality and abortion in cattle (4). This disease results in economic losses in the cattle industry, especially in beef-producing countries, such as the US, Argentina and other South American countries (5). Cfv is host-specific and shows tissue tropism, as they are restricted to the genital tract of cattle. Cfv resides persistently and asymptomatically in the epithelial crypts of the prepuce of bulls and is transmitted to cows by coitus with infected bulls or artificial insemination with contaminated semen (5).

In contrast to Cfv which has a niche preference for the bovine genital tract, Cff has a wide range of host distribution such as cattle, sheep and humans. Cff is transmitted through food and water ingestion and is commonly colonized in the intestinal tracts. However, Cff can also reach and infect the uterus through systemic infection, resulting in abortions in cows and sheep (6). Furthermore, Cff can infect other organs/tissues via bacteraemia, leading to conditions such as endocarditis, pericarditis, sepsis and meningitis (7).

1.3 Virulence factors

The virulence mechanisms underlying infection of *C. fetus* and its hosts are poorly understood. The best characterized virulence factor of *C. fetus* is the surface layer (S-layer), a protein structure covering the cell surface by surface array protein (Sap). The S-layer plays a significant role in evading the host's immune response through antigenic variation and by preventing C3b binding to the cell surface. Based on the type of *sap* genes, *C. fetus* can be either serotypes A, B, or AB. Cfv always contains *sapA* genes and can thus only be type A, whereas Cff could be type A, type B, or type AB (8,9).

Another virulence factor found in various gram-negative bacteria is the cytolethal distending toxin (Cdt), which induces cell death in the host. Cdt consists of three subunits, encoded by *cdtA*, *cdtB* and *cdtC* genes. The CdtA and CdtC subunits are responsible for binding to host cells, while CdtB subunits have DNase I activity, causing DNA damage to host cells. *C. fetus* presumably uses Cdt to disrupt the epithelial barrier, enabling the bacteria to penetrate and access the basolateral side of the cells (9,10).

Additionally, more potential virulence factors have been identified in the genome of *C. fetus*, including genes involved in adhesion and invasion (see Table 1) (9,11). However, current knowledge about these virulence factors could not explain the niche preference of *C. fetus*. As a result, cell culture-based studies are emerging to investigate host-pathogen interactions.

Table 1. Potential virulence factors involved in adhesion and invasion of C. fetus to host cells (9,11).

Gene	Protein	Adhesion/invasion
peb1	Putative binding component of an ABC transporter (PEB1)	Adhesion
cadF	Fibronectin binding protein	Adhesion
cmp	Major outer membrane protein (MOMP)	Adhesion
jlpA	Jejuni lipoprotein A (JlpA)	Adhesion
flaA and flaB	Flagellin protein (FlaA, FlaB)	Adhesion and invasion
VirB1 - VirB11 and VirD4	Type IV secretion system (T4SS)	Adhesion and invasion
ciaB	Campylobacter invasion antigen B (CiaB)	Invasion
iamABC	Invasion-associated marker ABC-transporter (iamABC).	Invasion

1.4 Adhesion and invasion

Epithelial cells are the first line of defence against pathogens, *C. fetus* must adhere and invade epithelial cells to colonize (12). The differences in host distribution and tissue tropism of *C. fetus* can be explained by its ability to adhere to and invade host-specific epithelial cells.

Several cell lines have been used to study the molecular mechanisms of attachment and internalization of *C. fetus*. Previous studies have demonstrated that both Cff and Cfv can adhere to and invade INT-407 cells (human embryonic intestinal epithelial cell line), Caco-2 cells (human colonic cell line) and ACH-3P cells (human choriocarcinoma cell line)(11,13–16). Other studies have shown the ability of Cff to invade HT-29/B6 cells (intestinal epithelial cell line) and the ability of Cfv to adhere to HeLa cells (cervical cancer cell line) and MBDK cells (canine kidney cell line) (17–19).

Primary cell cultures, which provide a better representation than immortalized cell lines, were also used to study the adherence and invasion of *C. fetus*. Campos-Múzquiz *et al.* have shown that Cff can adhere to and invade bovine endometrial epithelial cells, but does not have the capacity to survive within the cells (20). Furthermore, the study by Chiapparrone *et al.* demonstrated that Cfv can bind to primary bovine uterine and vaginal cells (18).

Nevertheless, the niche preferences of the two *C. fetus* subspecies have not been researched or explained by the above-mentioned studies. The primary aim of these studies was to examine the adhesion and/or invasion of *C. fetus*, rather than its niche preference. Therefore, most research has focused on only one *C. fetus* subspecies. For instance, Campos-Múzquiz *et al.* studied Cff, while Chiapparrone *et al.* focused on Cfv (18,20). Due to differences in methodologies, including variations in strains and cell types, it is impossible to directly compare the adhesion and invasion abilities of the two subspecies across different studies.

In studies where both subspecies were examined, direct comparisons between them were not made. For example, a figure in the study from Kienesberger *et al*. showed that Cfv has a lower invasion ability in Caco-2 and ACH-3P cells compared to Cff, although the significance of this

difference was not statistically tested (11). Moreover, they found that invasion was strain-dependent, with some strains being non-invasive, while one Cfv strain was 100 times more invasive than another. Thus, it remains unclear whether the differences in invasion abilities between the two subspecies are related to niche preference.

1.5 Air-liquid interface

An adequate in vitro model that mimics the *in vivo* environment for *C. fetus* is lacking. Studies using primary epithelial cell lines mentioned above were all tested with submerged cell cultures (18,20). Epithelial cells grown in submerged cultures may remain undifferentiated, while primary reproductive epithelial cells consist of multiple cell types, including basal cells, goblet cells and ciliated epithelial cells. Epithelial cells grown in submerged culture are unpolarized as well, whereas tight junctions, which maintain polarity, are crucial for the physiological barrier function. In short, the physiological conditions of submerged cell cultures are not representative of the epithelium of the reproductive tract (12,21).

Air-liquid interface (ALI) culture is an alternative to submerged cell culture by mimicking the native condition of reproductive epithelium. In contrast to submerged cell culture, epithelial cells in ALI culture are seeded on a porous filter support (Transwell insert). After reaching confluence, cells are apically exposed to the air (see Figure 1) (22). Cells receive their nutrients from the basolateral side through diffusion via the porous membrane. Under this condition, ALI promotes the formation of tight junctions, resulting in distinct baso-apical polarity. Moreover, introducing bacteria to the apical side of the cell provides a more natural pathogen exposure. Finally, ALI induces cell differentiation, in which mucus-producing goblet cells and ciliated epithelial cells can be differentiated (21,22).

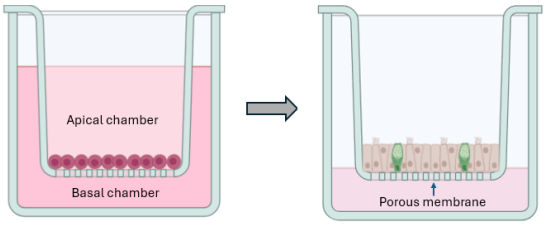

Figure 1. Schematic representation of air-liquid interface culture using epithelial cells*. The culture system consists of apical and basal compartments, separated by a porous membrane, on which epithelial cells are cultured. Once the epithelial cells reach confluence, the apical side is exposed to air, promoting cell differentiation (22).*

The objective of this study is to develop an ALI model using primary cells to investigate the niche preference of *C. fetus*. Epithelial cells in ALI culture mimic the native condition of reproductive epithelium by promoting polarization and differentiation, providing an *in vivo*-like environment for *C. fetus*. The niche preference of two *C. fetus* subspecies will be evaluated by comparing their attachment abilities to reproductive epithelial cells.

With this approach, the following research question will be addressed: How to develop an air-liquid interface model using primary epithelial cells to investigate the niche preference of *Campylobacter fetus*?

2. Materials and methods

2.1 Bacterial strains and culture conditions

Eight *C. fetus* strains used in this study are shown in Table 2. Included were strains of both subspecies, four well-characterized laboratory strains and four clinical strains. The bacterial strains were stored in nutrients broth with glycerol at -80°C. *C. fetus* was cultured on Columbia agar with sheep blood (BA plates) (Thermo Scientific, Oxoid, Germany) at 37°C for 48 hours under a microaerophilic atmosphere (6% O_2, 7,1% CO_2, 3,6% H_2, 83,3% N_2) using Anoxomat (Mart microbiology).

Optimal growth of *C. fetus* is necessary for the infection assays. Therefore, bacterial growth curves in different liquid media were made to determine the most suitable medium for *C. fetus* growth and its logarithmic (log) phases.

Table 2. Characterization of eight C. fetus strains used in this study.

Strain	Subspecies	Type of strain	Source
NCTC10842	Cff	Laboratory	Sheep, France
NCTC10354	Cfv	Laboratory	Bovine, UK
84-112	Cfv	Laboratory	Bovine, Argentina
82-40	Cff	Laboratory	Human, US
83	Cfv	Clinical	Bovine, New Caledonia
B0042	Cff	Clinical	Bovine, UK
925	Cff	Clinical	Bovine, Argentina
Ru-2009-08871-5 direct	Cfv	Clinical	Bovine, NL

Cfv = *Campylobacter fetus venerealis*; Cff = *Campylobacter fetus fetus*; UK = United Kingdom; US = United States; NL = The Netherlands

2.1.1 Culture of C. fetus in Dulbecco's Modified Eagle's Medium

Dulbecco's Modified Eagle's Medium (DMEM), one of the most used basal media for mammalian cell cultures, will be used for cell cultures (23). Since *C. fetus* will come into contact with DMEM during the infection experiments, it is necessary to know whether *C. fetus* grows properly in DMEM.

Colonies of the four laboratory *C. fetus* strains grown on BA plates were suspended in physiological saline solution to create a 5,0 McFarland suspension. The 5,0 McFarland suspension was measured with a Den-1 McFarland Densitometer (Grant Instruments, Cambridge, England). Subsequently, 100 µl of the 5.0 McFarland suspension was added to 25 cm^2 cell (T-25) culture flasks with canted neck and filter cap (Corning, AZ, US) containing 10 ml DMEM supplemented with 4,5 g/L D-glucose and L-Glutamine(Life Technologies, NY, US). The culture flasks were then incubated for 2-3 days at 37°C under microaerophilic conditions while shaking at 160 rpm. To determine the bacteria growth, serial dilution of the culture was plated and the numbers of colonies forming unit (CFU) were counted in triplicates at 0h, 4h, 22h, 28h, 46h and 52h. Similar replicates were performed to determine the optimal conditions for *C. fetus* growth in DMEM, including inoculation made with 100 µl of 0.5 McFarland suspension or incubation without shaking.

2.1.2 Culture of C. fetus in Heart Infusion Broth and Brain Heart Infusion Broth

Five to ten colonies of the four laboratory strains were transferred to T-25 culture flasks containing 15 ml Heart Infusion (HI) broth (BioTrading, Mijdrecht, The Netherlands) and Brain Heart Infusion (BHI) broth (Thermo Scientific). The culture flasks were then incubated for 2-3 days at 37°C in a microaerophilic atmosphere and shaking at 160 rpm. The growth of *C. fetus* in HI and BHI broth was analysed by measurement of the optical density at 600 nm (OD_{600}) at 0h, 4h, 22h, 28h, 45h and 52h using an Ultrospec 10 Cell density meter (Amersham Biosciences, Biochrom, Cambridge, England).

2.1.3 Culture of clinical C. fetus isolates in BHI broth

A 5,0 McFarland suspension was made and 100 µl of the 5.0 McFarland suspension was added to T-25 cell culture flasks containing 10 ml of BHI. The culture flasks were then incubated for 2-3 days at 37°C in a microaerophilic atmosphere and shaking at 160 rpm. The growth of *C. fetus* in HI and BHI broth was performed in triplicates and analysed by OD_{600} measurement and plate counting. For Cff, measurements were taken at 0h, 4h, 22h, 29h, 46h, and 52h, while for Cfv, they were taken at 0h, 6h, 23h, 30h, 47h, and 54h.

2.2 Primary epithelial cell culture conditions

2.2.1 Cell isolation of bovine uteri

Two whole bovine uteri were obtained from a slaughterhouse in NL, of which one of the uteri contained a foetus. The uteri were stored in cold Dulbecco's Phosphate-Buffered Saline without Ca and Mg (DPBS-/-; Capricorn Scientific, Germany) and transported with cool elements to the dirty ALI laboratory for one hour. Upon arrival, the bovine uteri were immersed in fresh ice for 3h. Two methods were used to isolate epithelial cells from the endometrium, cervix, placenta and foetal trachea (see Table 3).

2.2.1.1 Method 1

Incisions were made in the uterine horn walls, and fat and connective tissue were removed as much as possible. The endometrium of the uterine horn was then stripped and stored in cold DPBS-/- on ice. The endometrial strips were pinned onto a styrofoam lid to open and flatten the surface, the strips were further dissected into pieces of 1 cm x 4 cm and stored in cold DPBS-/- again. The dissected endometrial pieces were minced into small pieces of 1-3 mm in isolation medium which contained advanced DMEM/F-12 (Life Technologies), 100x L-alanyl-L-glutamine (Life Technologies), 1M Hydroxyethylpiperazine Ethane Sulfonic Acid (HEPES) buffer solution (Life Technologies) and 2% 10,000 U/mL Penicillin-Streptomycin (PenStrep; Life Technologies). The minced tissue was washed 3 times with DPBS-/- and Penstrep by centrifuging at 200×g for 5 minutes. Digestion medium which contains 10% collagenase type I-A (Sigma-Aldrich, Germany) and 90% isolation medium, was added to the minced tissue and incubated at 37°C while shaking at 160 rpm for up to 2 hours until the minced tissue had broken into smaller pieces. The digestion reaction was stopped by adding 10% fetal bovine serum (FBS), and the suspension was filtered with a 40 µm strainer.

The filter fraction contains epithelial cells, and the filtrate contains stromal fibroblasts, leukocytes and some endometrium stem cells. The filter fraction was placed on the petri dishes and washed with DMEM/F12. Both the filter fraction and the filtrate were then separately

transferred into collagen I/III-coated T-25 flasks or T-75 flasks with DMEM/F12, 10% FBS and cultured at 37°C in 5% CO_2. The cultured epithelial cells and stromal fibroblasts were monitored daily under the microscope until a sufficient number of cells had attached to the bottom of the flask. Once the attached cells had grown, the media was removed, and the cells were washed with warm Hanks' Balanced Salt Solution without Ca and Mg (HBSS-/-; Life Technologies). The cells were then incubated with 1,5 ml TrypLE(Life Technologies) at 37°C until the cells were visibly detached. Old media and HBSS-/- were then added to inhibit the working of trypsin. The cell suspension was filtered through a 40 µm strainer and centrifuged at 200×g for 5 minutes. The resulting pellet was centrifuged again at 400×g for 5 minutes in warm HBSS-/-. Finally, the pellet was diluted in cryopreservation medium (DMEM/F-12, dimethyl sulfoxide (DMSO; Life Technologies) and FBS) to achieve a concentration of 0,5-1 million cells/ml and stored initially at -80°C before being transferred to liquid nitrogen storage.

2.2.1.2 Method 2

Incisions were made in the uterus and fat and connective tissue were removed as much as possible (see Figure 2A). The epithelial tissue of the uterine horn, cervix, placenta and foetal trachea were excised and pinned onto a styrofoam lid to open and flatten the surface (see Figure 2B). The epithelial tissue was further dissected and incubated in digestive medium (protease/DNase solution and isolation medium) at 4°C for 20h to 48h (see Figure 2C). Secondly, the epithelial tissues were scrapped in DPBS-/- into very small pieces. To inactivate proteases, DPBS-/- was added to the scrapped tissue to a final concentration of 10%. The suspension was further centrifuged at 200×g for 5 minutes. The resulting pellet was resuspended in HBSS-/-, and the suspension was filtered through a 40 µm strainer. The filtrate was then centrifuged at 200×g for 5 minutes. Finally, the pellet was diluted in cryopreservation medium to achieve a concentration of 0,5-1 million cells/ml and stored in an 80°C freezer, followed by liquid nitrogen storage transfer.

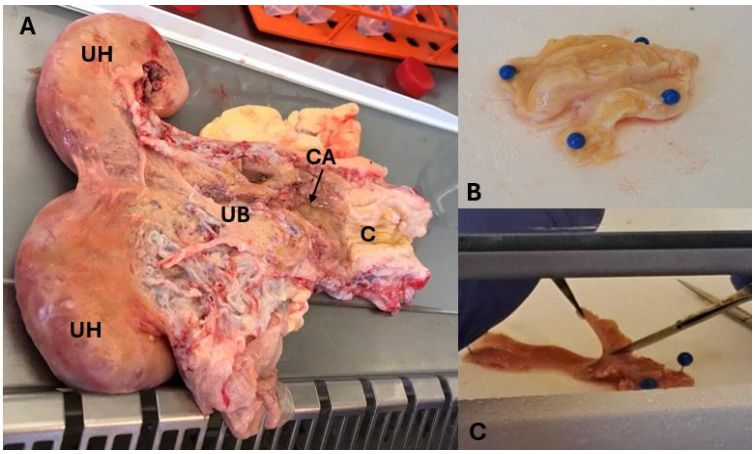

Figure 2. Images of bovine uterus used for cell isolation. *A: Bovine uterus with an incision and its anatomy: UH uterine horn, UB uterine body, CA Caruncle, C cervix; B: Cervix tissue pinned on styrofoam; C: Stripped endometrium*

Table 3. Isolated bovine epithelial cells from multiple tissues using different methods.

	Uterine horn (cow 1)	Uterine horn (cow 2)	Cervix (cow 1)	Cervix (cow 2)	Placenta (cow 2)	Foetal trachea (cow 2)
Method 1	x	x				
Method 2		x	x	x	x	x

Cow 1: nonpregnant cow; cow 2: pregnant cow carrying a foetus.

2.2.2 Thawing and seeding of bovine cervix epithelial cell

The frozen bovine cervical epithelial cells stored in cryovials were rapidly thawed in a 37°C water bath. The thawed bovine cervical epithelial cells were then cultured in Bronchial Epithelial Cell Growth Medium (BEGM) on collagen type I and III-coated T-25 flasks at 37 °C in a humidified atmosphere of 10% CO_2. BEGM growth medium was changed every 2-3 days. BEGM was prepared using LHC basal medium supplemented with additives as described by Gultom *et al.* (24).

2.2.3 Thawing and seeding of porcine cervix epithelial cell

Frozen primary porcine cervical epithelial cells from a 10-week-old pig provided by the Department of Pathology, were cultured in BEGM on collagen type I and III-coated T-75 flasks at 37 °C in a humidified atmosphere of 10% CO_2. BEGM was replaced every 2-3 days until the cells had reached 80-90% confluence.

2.2.4 Subculture of porcine cervix epithelial cell

The proliferated epithelial cells were washed twice with HBSS-/- after they had reached 80-90% confluence. The attached cells were dissociated using 5 ml of TrypLE and incubated for 5-10 min at 37°C in a humidified 10% CO_2 atmosphere. The dissociated cells were washed with HBSS-/- to remove residual trypsin and then resuspended in 6 ml BEGM medium. To generate a liquid-liquid interface, the basolateral side of the culture plates was filled with 500 µl of BEGM medium and 200 µl of the diluted cell suspension was seeded on collagen type IV-coated 6,5 mm Transwell inserts (Corning, #3470) at a density of 1,0–1,5 x 10^5 cells per insert. The cells were incubated in a humidified atmosphere of 10% CO_2 and the BEGM growth medium was changed every 2-3 days.

Once the cells reached confluence on the inserts, as observed under a microscope, the transepithelial electrical resistance (TEER) of the cells was measured using an Ohm meter to confirm the integrity and permeability of the cellular monolayer. A TEER value above 500 Ω/cm² indicated that the cells were ready to differentiate (24). The BEGM medium was then replaced with ALI medium, prepared as described by Gultom *et al*., and the cells were kept at a liquid-liquid interface for two days (24). After two days, the apical medium was removed to establish ALI. The basolateral medium was changed every 2–3 days to prevent acidification.

2.3 Bacterial staining

2.3.1 BacLight bacterial stain

Overnight (O/N) cultures of *C. fetus* in BHI were diluted to 1 x 10^4 CFU/ml in DPBS-/-. The bacterial cells were stained by adding 1 µl of BacLight bacterial stain (Thermo Scientific) at concentrations of 100 µM, 250 µM or 500 µM to 1 ml bacteria suspension. Afterwards, *C. fetus* was incubated in the dark for 15 minutes at room temperature (RT). To determine whether a wash step is required for BacLight staining to remove excess stain, one of the two samples stained with 250 µM BacLight was washed with DPBS-/- The stained bacteria cells were then visualised using fluorescent microscopy.

2.3.2 CFSE staining

Carboxyfluorescein succinimidyl ester (CFSE; Biolegend, CA, US), a fluorescent cell staining dye that is commonly used to track cell proliferation, were used to stain *C. fetus*. *Escherichia coli* (*E. coli*) strain TOP 10 was used as a control since it has been confirmed that CFSE labels with this bacterium. O/N cultures of Cff 925 in BHI, 24-hour cultures of *E. coli* in BHI and colonies of Cff strain B0042 grown on BA plates resuspended in BHI were standardized to an OD_{600} of 1,0. Cells were collected by centrifuging 1 ml of each culture at 3000 xg for 10 minutes and washed once with 1 ml DPBS-/-. The pellet was then stained with 250 µl of 60 µM CFSE working concentration in DPBS-/-. Afterwards, bacterial cells were incubated in the dark at 37°C for 45-60 minutes. Following incubation, bacterial cells were washed three times by centrifuging at 3000 xg for 10 minutes using BHI and resuspended in 1 ml BHI.

To investigate the influence of DPBS-/- incubation on *C. fetus*, O/N cultures of Cff strain 925 in BHI were stained with CFSE and incubated for 1 hour in BHI or DPBS-/-. After incubation, the pellet of bacterial suspension was resuspended in BHI and incubated overnight. The CFSE-stained O/N cultures were then labelled with Hoechst to determine whether the cells had lost their fluorescent signal due to cell division. All samples were visualized using fluorescent microscopy.

2.4 Infection assay

Three-day-old colonies of Cff strain 925 grown on BA plates were resuspended in BHI and stained with CFSE. The epithelial cells were apically infected with 100 µl of the stained bacterial suspension in DPBS-/- or BHI at a multiplicity of infection (MOI) of 50 or 500 (see Figure 3). Cells were then incubated for 1 hour at 37°C in a microaerophilic atmosphere with 10% CO_2. The bacterial suspension was removed after incubation, some of the inserts were directly fixated with 4% paraformaldehyde (PFA) in DPBS-/- (Thermo Scientific), while others were further incubated overnight and fixated afterwards. The fixated cells were then stored in DPBS-/- at -4°C and viewed by fluorescent microscopy. The remaining Cff suspensions in BHI were quantified by plate counting.

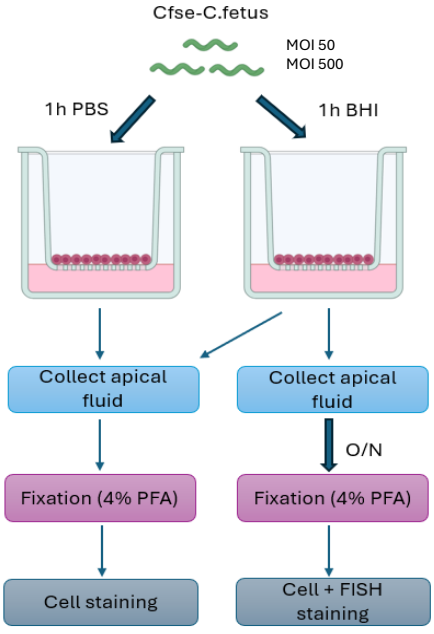

Figure 3. General steps of the infection assay. *Porcine cervical epithelial cells were infected with CFSE-stained Cff strain 925 at MOI 50 or 500 and incubated for 1h in DPBS-/- or BHI. Apical fluids were then collected and some of the samples were further incubated O/N, while others were fixated with 4% PFA and stained for cell type confirmation. O/N incubated samples were fixated and stained as well, with an additional FISH staining.*

2.5 Fluorescent assay

2.5.1 Cell staining

For studying cell integrity, cell nuclei were stained with 4′,6-diamidino-2 phenylindole (DAPI; InVitrogen. MA, US), the cell surface with Biotinylated Maackia amurensis lectin II (MALII; Vector labs, CA, US) and cilia of ciliated epithelial cells with anti-beta-tubulin (BTUB; ITK diagnostics, The Netherlands). Samples infected at MOI 50 infected with 1h DPBS-/-, 1h BHI or O/N BHI incubation were first blocked

with blocking solution (1% bovine serum albumin and 0.3% Triton X-100 in DPBS-/-) for an hour at RT. Secondly, cells were apically stained with primary antibodies (mouse anti-BTUB and biotin-MALII) diluted 1:100 in blocking solution and incubated for an hour at RT. Afterwards, the cells were washed three times for 5 minutes with DPBS-/- and incubated with secondary antibodies (Goat anti-mouse IgG- Alexa Fluor 568 (InVitrogen), streptavidin-Alexa Fluor 647 (InVitrogen)) diluted in 1:100 and DAPI diluted in 1:2500 for 30 min at RT. After three wash steps with DPBS-/- for 5 minutes, the filter of the well was placed with face upwards on a glass slide and mounted with ProLong Diamond Antifade Mountant (InVitrogen). Images were acquired using a confocal microscope (Leica microsystems, Wetzlar, Germany).

2.5.2 Fluorescent in situ hybridization staining

Fluorescent in situ hybridization (FISH) staining was used to determine the presence of *C. fetus* after O/N incubation, as the fluorescent signal of CFSE will become weaker after bacterial cell divisions. Fixated Cells that were incubated O/N in BHI at MOI 50 or 500 were stained with FISH probe EUB338-A488 (Integrated DNA Technologies, IA, US). The cells were first washed thrice in DPBS+/+ (Sigma-Aldrich) and then incubated with 50 mM NH_4Cl in DPBS-/- for 10 min. Afterwards, cells were incubated with 6 ng/µl of the FISH probe in hybridization buffer (0.9 M NaCl, 20 mM tris pH 7.5, 0.1% SDS and 20% formamide) for 2h at 45°C in a humidified chamber. Thereafter, cells were washed twice with DPBS+/+ and stained with DAPI, MALII and BTUB as previously described.

2.6 Genome analysis

2.6.1 Phylogenetic tree and single nucleotide polymorphism analysis

Phylogenetic analysis of 50 *C. fetus* genomes (see Appendix 1), including the genomes from the eight *C. fetus* strains used in this study, was performed using Parsnp v1.2 with the parameters: -c, -d, -r !, -o. The code for Parsnp can be found at https://github.com/marbl/parsnp and in Appendix 2. Furthermore, the *cdt* and *sap* genes of the genomes were annotated using Bakta v1.4 with the default settings (25). These annotated genes were aligned against the reference genome, using BLASTp with Cut-off values of > 97% identity and > 95% query coverage per subject, or BLASTn with cut-off values of > 97% identity and an alignment length > 18 (26).

2.6.2 Carbohydrate-active enzymes analysis

The capability of enzymatically degrading mucin O-glycan by glycoside hydrolases (GH) was examined by identifying the carbohydrate-active enzymes (CAZymes) in *C. fetus genomes*. CAZymes of *C. fetus* were annotated from nucleotide sequences using dbCAN3 meta server and were identified when they were annotated by at least two out of three annotation tools (HMMER: dbCAN, DIAMOND: CAZy, and HMMER: dbCAN_sub) (27). The genome sequence of *Akkermansia muciniphila* 22S00963 was used as a control.

3. Results

3.1 *C. fetus* culture conditions

3.1.1 Culture of laboratory C. fetus strains in DMEM

Laboratory *C. fetus* strains were cultured in DMEM and their growth curves are shown in Figure 4. The initial inoculum concentration for the four *C. fetus* strains was consistent, approximately 1 x 10^7 CFU/ml. The number of living bacterial cells of all *C. fetus* strains, except Cfv strain NCTC10354, declines up to 22 hours in DMEM. After 22 hours, all strains except Cfv strain NCTC10354 entered the log phase, showing an exponential increase of cells of max 1 x 10^6 CFU/ml. In contrast to other strains, Cfv strain NCTC10354 did not enter the log phase and decreased up to 5 x 10^3 CFU/ml.

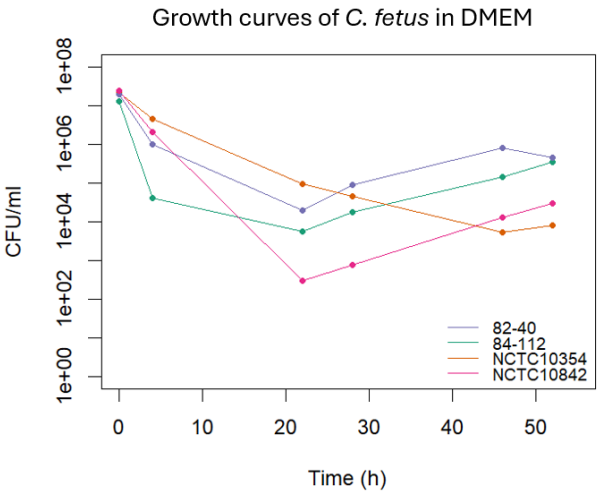

Figure 4. Growth curves of four laboratory C. fetus strains grown in DMEM at 37◦C, 160 rpm for 52 h. *The growth of C. fetus in DMEM was measured at 0h, 4h, 22h, 28h, 46h and 52h using plate counting*

Similar experiments were performed with a few modified conditions, including a lower starting inoculum using 100 µl of 0.5 McFarland suspension and a comparison between static and shaken incubation. However, despite these changes, the overall growth patterns of the growth curves remained similar across trials, no significant increase in bacterial growth was observed between the growth curves (see Appendix 3). Since *C. fetus* did not grow well in DMEM, an alternative medium is needed to achieve optimal bacterial growth. Therefore, the growth of *C. fetus* in HI and BHI was examined.

3.1.2 Culture of laboratory C. fetus strains in HI broth and BHI broth

Laboratory *C. fetus* strains were cultured in BHI and HI, and their growth curves are shown in Figure 5. All *C. fetus* stains remain in the lag phase for the first 4 hours, followed by a log phase. After 28 hours, all strains, except Cfv strain NCTC10354 grown in HI, reached the stationary phase. Additionally, it is shown that Cff grows better in BHI and HI than Cfv. Cff reaches an $OD_{600} > 1$ in both BHI and HI, while Cfv strains reach an OD_{600} of maximum 0,59 and 0,70 in BHI and 0,43 and 0,28 in HI.

Due to inconsistencies of the initial inoculum concentrations, Cff strain 82-40 and Cfv strain NCTC10354 in BHI have slightly higher initial inoculum concentrations with an $OD_{600} = 0,08$, whereas other inoculums in HI and BHI were consistent with an $OD_{600} = 0.01$ or 0.02. Therefore, Cff strain 82-40 and Cfv strain NCTC10354 will be excluded from the comparison. The growth curves show that both Cff strain NCTC10842 and Cfv strain 84-112 strain reach a higher OD_{600} in BHI than in HI. The results indicate that *C. fetus* grows well in both media, with BHI being the better option. Therefore, BHI will be used to culture clinical strains to determine if they also grow well in this medium.

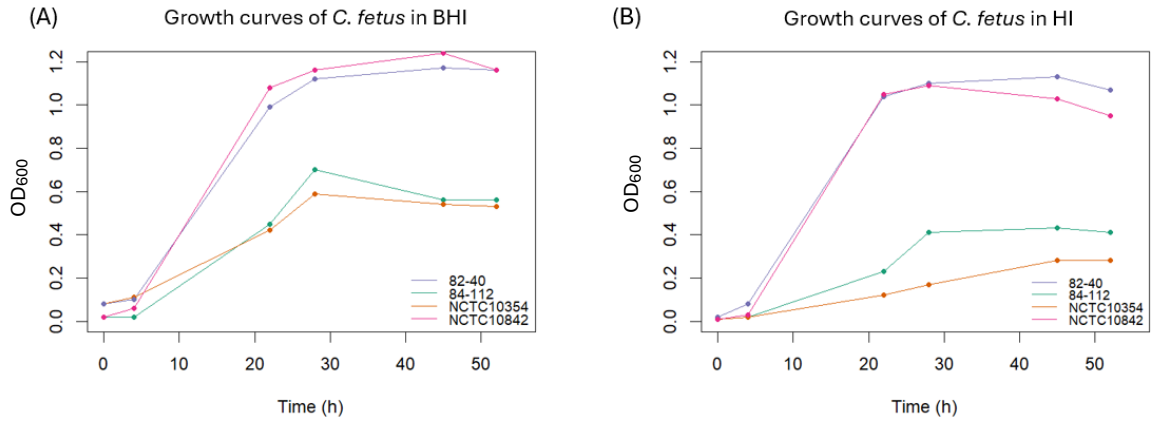

Figure 5. Growth curves of four laboratory C. fetus strains grown in BHI and HI at 37◦C, 160 rpm for 52 h. *A: Growth curves of C. fetus in BHI; B: Growth curves of C. fetus in HI. The growth of C. fetus in HI and BHI broth was analysed by OD_{600} measurement at 0h, 4h, 22h, 28h, 45h and 52h.*

3.1.3 Culture of clinical C. fetus strains in BHI broth

Clinical *C. fetus* strains were cultured in BHI, with experiments performed in triplicate to ensure reliable results. Growth curves of clinical *C. fetus* isolates in BHI are shown in Figure 6. By comparing OD_{600} -values with plate counting, it is considered that an OD_{600} = 0,01 for *C. fetus* suspension in BHI is approximately 1 x 10^7 CFU/ml, OD_{600} = 0,10 to 1 x 10^8 CFU/ml and OD_{600} = 1,0 to 1 x 10^9 CFU/ml. The initial inoculum concentration for the four *C. fetus* strains was consistent, approximately 1 x 10^7 CFU/ml with OD_{600} = 0,00-0,01.

All *C. fetus* strains remained in the lag phase for the first 4-6 hours, followed by a log phase. Cfv strains reached the stationary phase after 30 hours and Cff strain 925 after 46 hours. Cff strain B0042 did not reach the stationary phase but entered the death phase directly after the log phase. It is shown that Cfv strain ru-2009-08871-5-direct and the two Cff strains grow well in BHI, reaching an OD_{600} > 0,77. In contrast, Cfv strain 83 only reached an OD600 of 0,26, though this corresponds to approximately 3 x 10^8 CFU/ml. Hence, BHI is a suitable medium for culturing *C. fetus*, with the bacteria in the log phase (between 6 and 30 hours) being ideal to use in infection assays.

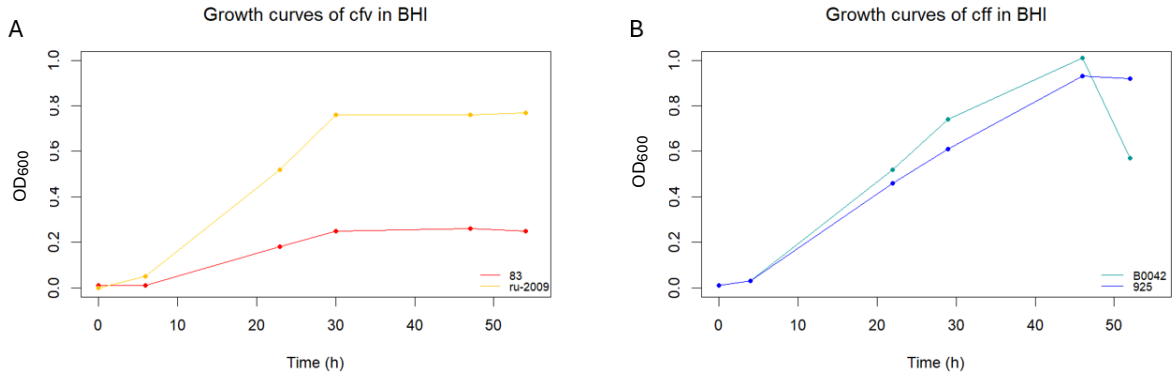

Figure 6. Growth curves of four clinical C. fetus strains grown in BHI at 37◦C, 160 rpm for 52 h or 54 h. *A: Growth curves of* Cfv *strains in BHI; B: Growth curves of* Cff *strains in BHI; The growth of C. fetus in BHI broth was analysed by measurement of OD_{600}. For Cff, measurements were taken at 0h, 4h, 22h, 29h, 46h, and 52h, while for Cfv, they were taken at 0h, 6h, 23h, 30h, 47h, and 54h.*

3.2 Bacterial staining

3.2.1 BacLight bacterial stain

Four laboratory *C. fetus strains* were stained with BacLight staining for detecting *C. fetus* cells and subsequently visualised using fluorescent microscopy. Signals of BacLight staining were not clearly visible in any samples with varying concentrations of BacLight stain. Additionally, no difference in signal clarity was observed between samples stained with 250 µM BacLight, regardless of whether a washing step was included. Since the signals of BacLight-stained *C. fetus* were not clearly visible, BacLight staining cannot be used to detect *C. fetus* in infection assays. Therefore, CFSE staining will be tested as an alternative.

3.2.2 CFSE staining

E. coli, Cff strains B0042 and 925 were stained with CFSE and visualised by fluorescent microscopy. CFSE-stained *E. coli* and Cff exhibited a clear green signal under the fluorescent microscope (see Figure 7).

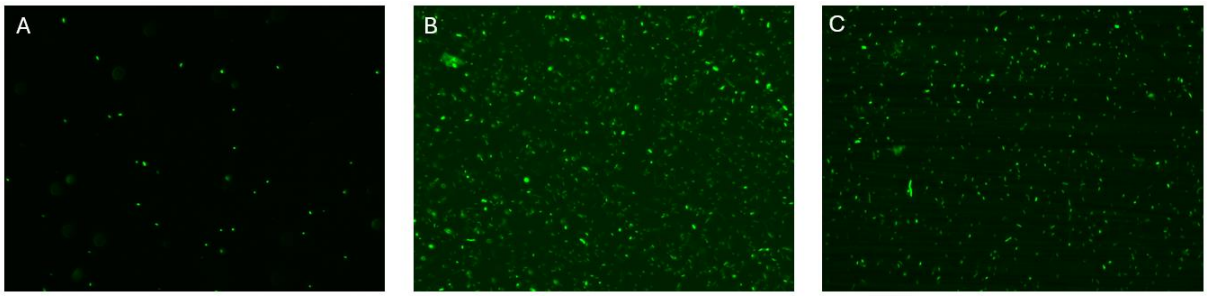

Figure 7. CFSE-stained E. coli, Cff strains B0042 and 925 visualised by fluorescent microscopy. *A: 24-hour culture of E. coli in BHI stained with CFSE; B: Cff strain B0042 resuspended in BHI stained with CFSE; C: O/N culture of Cff strain 925 in BHI stained with CFSE*

To investigate the effect of DPBS-/- incubation on *C. fetus*, CFSE-stained Cff strain 925 was incubated for 1 hour in either BHI or DPBS-/-. No significant differences in fluorescent signals or cell numbers were observed between the two conditions. Consequently, these bacterial suspensions were further incubated O/N in BHI, followed by Hoechst staining. A higher number of *C. fetus* cells, incubated for 1 hour in BHI followed by an O/N incubation, exhibited Hoechst signals compared to CFSE signals, of which a small part of cells exhibited both signals (see Figure 8A, B, and C).

In contrast, most of the *C. fetus* cells incubated for 1 hour in DPBS-/- followed by an O/N incubation, exhibited both Hoechst and CFSE signals, although the CFSE signal was noticeably weaker in some cells (see Figure 8D, E, F). This suggests that *C. fetus* continuously incubated in BHI underwent more cell division, losing most of the CFSE signal, while those incubated in DPBS-/- for 1 hour underwent less division. In conclusion, CFSE is an effective stain for *C. fetus*, although some signal loss may occur after multiple cell divisions.

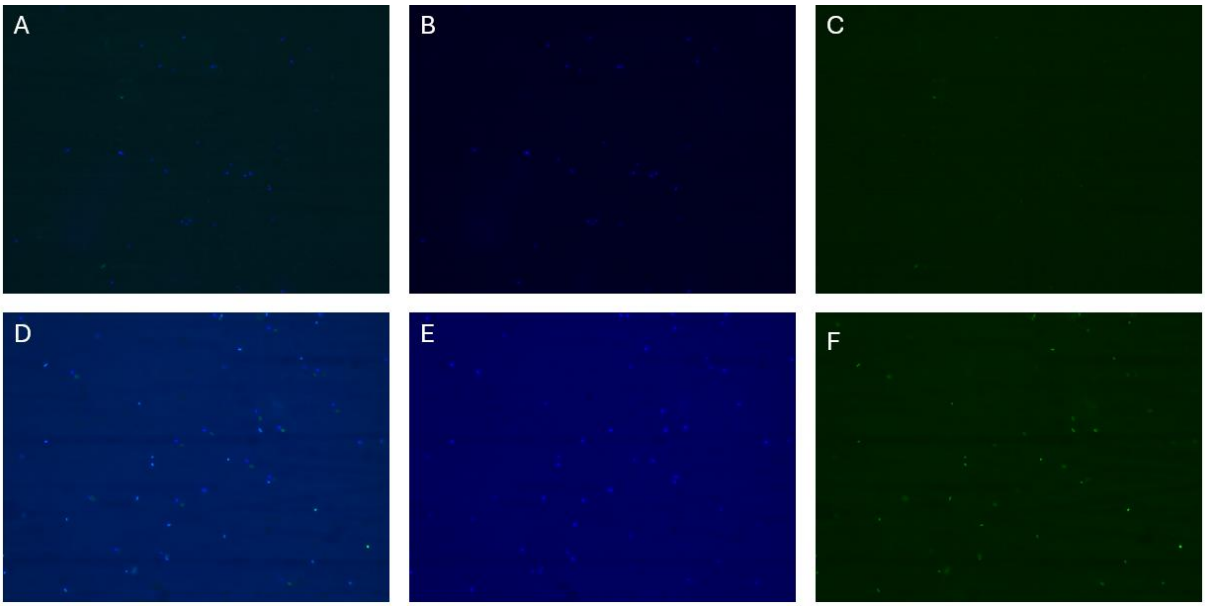

Figure 8. CFSE-stained Cff strain 925 after O/N incubation in BHI followed by Hoechst staining. *A-C: C. fetus incubated in BHI for 1 hour, followed by O/N incubation in BHI; D-F: C. fetus cells incubated in DPBS-/- for 1 hour, followed by O/N incubation in BHI; A and D: Overlapping Hoechst and CFSE signals of Cff strain 925; B ad E: Hoechst signals of Cff strain 925; C and F: CFSE signals of Cff strain 925; All samples were visualised using fluorescent microscopy*

3.3 Culture of isolated cervical epithelial cells

Isolated bovine cervical epithelial cells were cultured in BEGM in T-25 flasks coated with collagen types I and III. The epithelial cells did not proliferate well enough to reach 80-90% confluence and could not be used for further subculturing. Given the time and materials, swine cervical epithelial cells were cultured for the infection assay.

Swine cervical epithelial cells were cultured under the same conditions as the bovine cervical cells, reaching 80-90% confluence after two weeks. A total of 20 inserts were seeded. After the cells were maintained at ALI, ciliated epithelial cells with movement were observed under the microscope, but no mucus production was detected. The porcine cervical epithelial cells began detaching at the edges of the inserts after one week in differentiation medium. Eight inserts, showing less detachment, were selected for a pilot infection assay.

3.4 Infection assay

Three-day-old Cff strain 925 grown on BA plates were resuspended in BHI due to the sudden detachments of epithelial cells. Plate counting of this *C. fetus* suspension showed that the MOI used in the infection assay was 50 and 500.

3.4.1 Adherence of C. fetus on porcine cervix epithelial cells

A pilot infection assay was performed on porcine cervix epithelial cells. Porcine cervical epithelial cells were infected with Cff strain 925 at an MOI of 50 or 500 and incubated for 1 hour in either DPBS-/- or BHI. Samples were then analysed immediately or following overnight infection. Signals from CFSE staining were not detected using a fluorescent microscope. Therefore, cells were stained and analysed using a confocal microscope (see Figure 9).

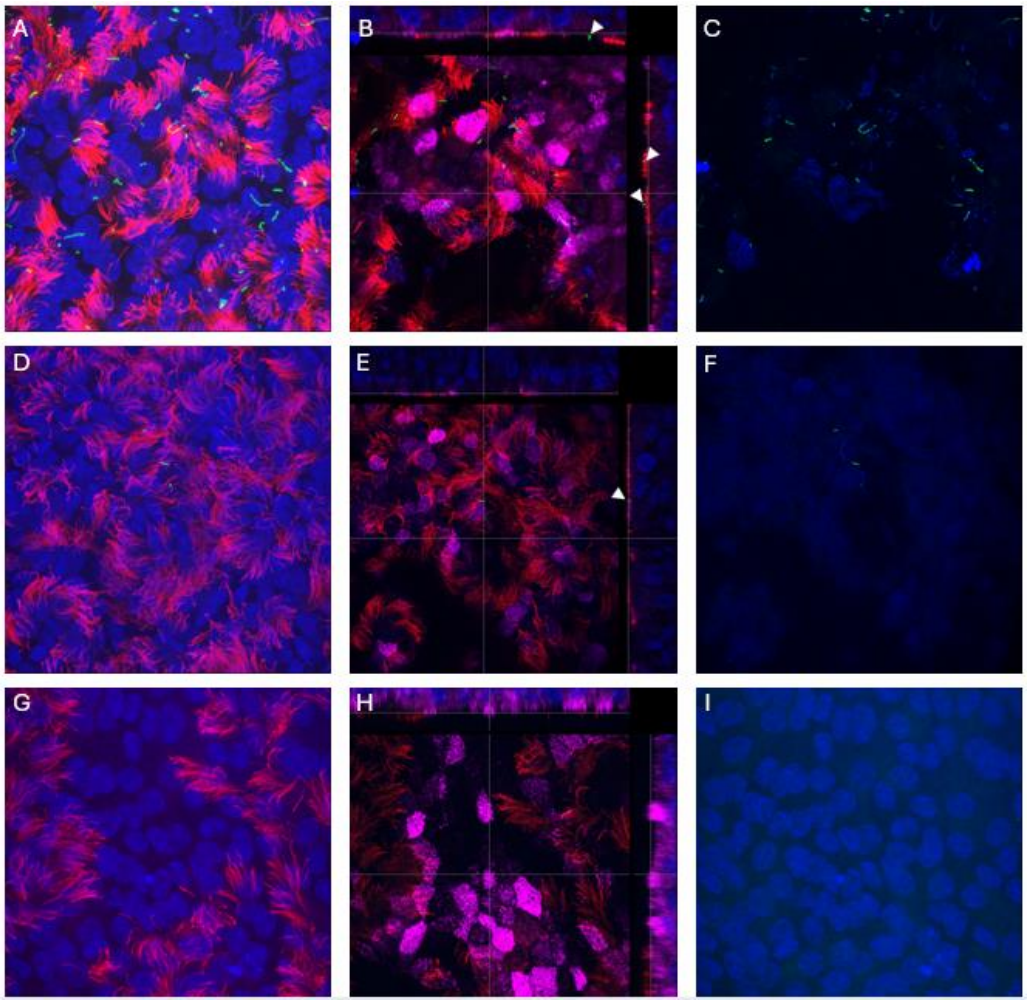

Figure 9. Porcine cervical epithelial cells infected with Cff strain 925 visualised using confocal microscopy.
Red: anti-BTUB-stained cilia; Blue: DAPI-stained cell nuclei; Purple: MALII-stained cell surface; Green: CFSE-stained Cff*;*
A-C: Porcine cervical epithelial cells infected with MOI 50 and incubated for 1 hour in BHI;
D-F: Porcine cervical epithelial cells infected with MOI 50 and incubated for 1 hour in DPBS-/-;
G-I: Porcine cervical epithelial cells infected with MOI 50 and incubated for 1 hour in BHI followed by O/N incubation;
A, D, G: Max projection of all channels; B, E, H: Overlay of the cell layers, the arrows point the location of C. fetus; C, F, I: Overlap of DAPI and CFSE signals

Ciliated cells, stained with anti-BTUB, were found on the first cell layer in all cell inserts (see Figures 9 and 10), confirming cell differentiation has occurred. Adhered *C. fetus* was observed in epithelial cells infected at MOI 50 and incubated for 1 hour in either BHI or DPBS-/- (see Figures 9A and 9D). A higher number of *C. fetus* cells were found in the sample incubated in BHI compared to DPBS-/-. Additionally, *C. fetus* cells were stained with DAPI beside the nucleus of epithelial cells. An overlap of DAPI and CFSE signals showed that some DAPI-stained *C. fetus* cells lacked CFSE signals, with more DAPI-stained *C. fetus* found in the BHI-incubated sample than in the DPBS-/- sample.

In contrast, no *C. fetus* was detected in epithelial cells infected at MOI 50 and incubated for 1 hour in BHI followed by overnight incubation. Finally, the location of *C. fetus* on the epithelial cell layers is indicated by arrows in figures 9B and 9E, showing the bacteria situated on the surface of the epithelial layer, near the cilia.

3.4.2 Adherence and invasion of C. fetus on porcine cervix epithelial cells

C. fetus was not detected in samples infected with Cff strain 925 at a MOI of 50 or 500 and incubated for 1 hour in BHI, followed by overnight incubation. To confirm the absence of *C. fetus*, samples were stained with the FISH probe EUB338-A488.

Weak FISH signals for *C. fetus* were detected in both samples (see Figure 10), with one bacterial cell in the MOI 500 sample showing a clear signal. One bacterial cell on the sample infected with MOI 50 was located on the underside of the cell layer, suggesting potential invasion. In contrast, the location of *C. fetus* in the MOI 500 sample was not visible, and no DAPI-stained *C. fetus* cells were detected in either sample. Finally, the MOI 500 infection resulted in fewer remaining cells on the inserts compared to the MOI 50 sample, suggesting that infection at MOI 500 caused more cell damage.

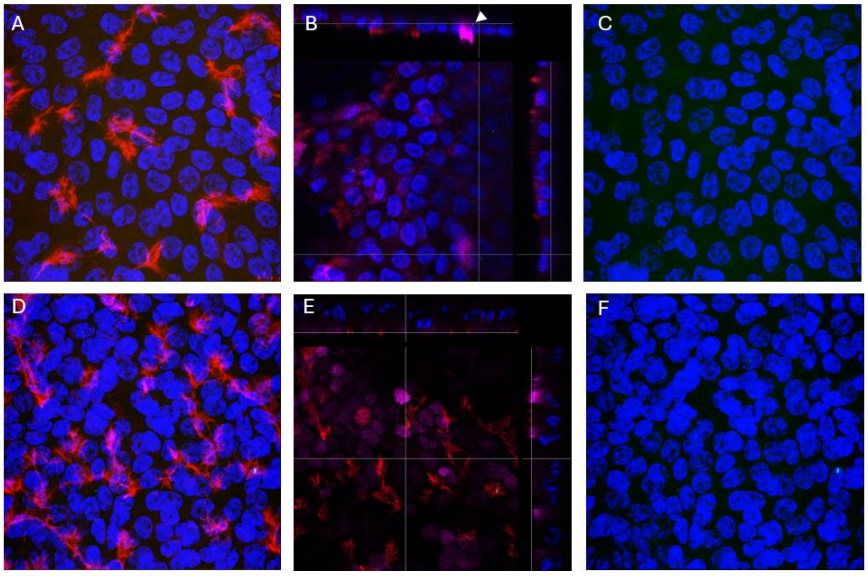

Figure 10. Porcine cervix epithelial cells infected with Cff strain 925 visualised using confocal microscopy.
Red: BTUB-stained cilia; Blue: DAPI-stained cell nucleus; Purple: MALII-stained cell surface; Green: FISH-labelled Cff*;*
A and G: Max projection of all colours; B and E: overlay of cell layers; C and I: overlap of DAPI and CFSE signals;
A-C: Cells infected with MOI 50 and incubated for 1 hour in BHI followed by O/N incubation;
D-F: Cells infected with MOI 500 and incubated for 1 hour in BHI followed by O/N incubation

3.5 Genome analysis

3.5.1 Phylogenetic tree and analysis of virulence genes

The genome sizes of the 50 *C. fetus* genomes range from 1.724.540 bp to 2.071.080 bp (see Appendix 1). The phylogenetic tree of these strains is shown in Figure 11. The phylogenetic tree shows multiple clusters, with their serotypes determined by the type of *sap* genes they have. The clusters outlined in purple represent serotype A, those in orange represent serotype AB, and those in green represent serotype B.

All 50 *C. fetus* strains contain *cdtA, cdtB*, and *cdtC* genes. However, compared to the reference genome, 19 of these strains have mismatches in the *cdtA* gene (see Appendix 4 and Figure 11).

Among these, 10 strains have a single nucleotide polymorphism (SNP) at p.V267A, six strains at p.H91Y, one strain at p.D49E, one strain at p.L77P, and one strain shows mutations at both p.L77P and p.G115D. Furthermore, 15 strains show mismatches in the *cdtB* gene, with three strains carrying both SNPs at p.V168I and p.A209T, and 12 strains carrying p.M61. In the *cdtC* gene, 19 strains show mismatches, with 10 strains having both p.I6T and p.P58S, and nine strains carrying p.S67A. These types of SNPs were found within clusters, with strains in each cluster originating from diverse hosts, including ovine, bovine, equine, and human. Finally, all these SNPs were present exclusively in the Cff strains and not in the Cfv strain.

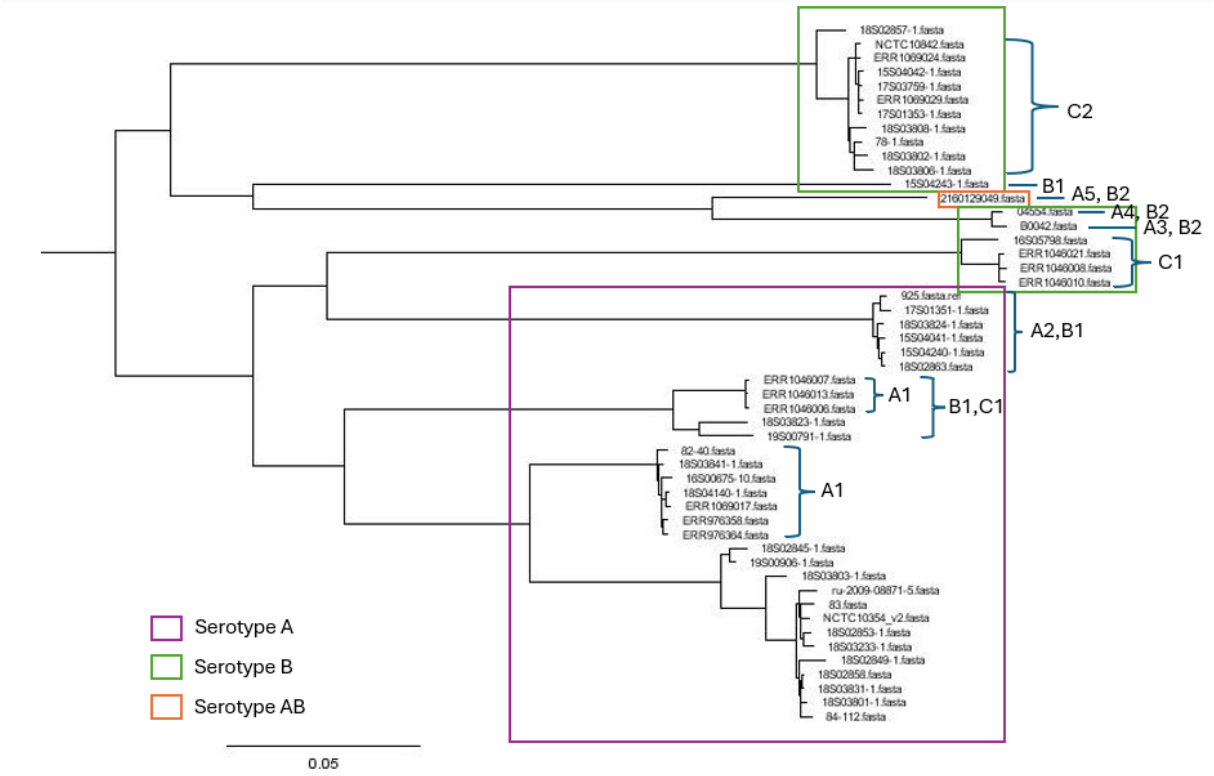

Figure 11. Phylogenetic tree of 50 C. fetus strains using program Parsnp v1.2. *Clusters outlined in purple represent C. fetus with serotype B, in orange represent serotype AB, and in green represent serotype A. SNPs found in cdtA gene: A1 = p.V267A, A2= p.H91Y, A3 = p. L77P and p.G115D, A4 = p.L77P and A5= p.D49E; SNPs found in cdtB gene: B1= p.M6I, B2= p.V168I and p.A209T; SNPs found in cdtC gene: C1= p.S67A, C2= p.I6T and p.P58S; Cdt and sap genes were annotated using Bakta v1.4, these genes were aligned against the reference genome using BLASTp and BLASTn.*

3.5.2 CAZyme analysis

Fifteen types of CAZymes were identified across all 8 *C. fetus* strains in this study (see Appendix 5). The total number of open reading frames (ORFs) in the Cff strains ranges from 33 to 37, while in the Cfv strains, it ranges from 36 to 38. This variation in ORF numbers is due to differing gene copy numbers for the proteins GH23, CE4, GT2, GT4 and GT9. Only three GHs (GH23, GH73, and GH3) were detected, indicating a limited capability of *C. fetus* to degrade mucin O-glycans. Additionally, *Akkermansia muciniphila* 22S00963 was found to have 146 ORFs, supporting the accuracy of the methods used for CAZyme analysis.

4. Discussion and conclusion

The aim of this research is to develop an ALI model using primary cells to investigate the niche preference of *C. fetus*. However, the model was not established due to several challenges that were encountered during this study. Fortunately, some key challenges were identified, and these can be addressed and modified in future studies to successfully set up a fully functional model and explore the niche preference of *C. fetus*.

4.1 Bacterial culture conditions

Different media were tested to determine the optimal culture conditions for *C. fetus*, with BHI proving to be the best option. In BHI, *C. fetus* entered the log phase between 6 and 30 hours. Generally, Cfv grows slower on BA plates compared to Cff and this phenomenon is also observed in BHI and HI. However, no difference was found between the growth of the two subspecies in DMEM, as both subspecies grew poorly in DMEM. Most *C. fetus* cells in DMEM died within the first 20 hours, but the number of living cells increased after 22 hours. The low growth rate was expected due to the limitations of nutrients in DMEM. Since Campylobacter species lack 6-phosphofructokinase, *C. fetus* can only conserve L-glutamine in DMEM as an energy source (28). Thereby suggesting that *C. fetus* need to activate the glutamine metabolic pathway to survive and divide in DMEM. Moreover, iron has been shown to play an important role in the metabolism and gene regulation of *Campylobacter coli* (*C. coli*) and *Campylobacter jejuni* (*C. jejuni)* (29). Further studies using defined media could determine which amino acids, citric acid cycle intermediates and other minerals, such as iron, are crucial for the growth of *C. fetus* and whether these requirements differ between subspecies. By adding these crucial nutrients that are lacking in DMEM, *C. fetus* might grow better in DMEM, which is the preferred incubation medium for epithelial cells.

4.2 Isolation methods for epithelial cells from bovine uterus

The proliferation of isolated bovine cervical epithelial cells took longer than 4 weeks, potentially leading to excessive passages and consequently, loss of cell differentiation activities. This issue was predominantly caused by low cell viability (see Appendix 6). The bovine epithelial cells were isolated 4 hours after the uterus was removed from the cow and incubated in cold DPBS-/-, which may have contributed to the reduced viability. Another study which has compared isolated cells from different transport conditions has also highlighted that transport conditions are a crucial factor for cell viability (30). In the future, isolating the epithelial cells immediately at the slaughterhouse would be ideal, although a travel time of one hour to the laboratory should not significantly affect cell viability.

Mucus release by goblet cells is a challenge in isolating primary uterine epithelial cells. Two methods were tested to isolate epithelial cells from the endometrium, cervix, placenta, and foetal trachea. In method 1, minced tissues were incubated in a digestion medium containing collagenase I for 1 hour, while in method 2, tissues were incubated for 20h to 48h in a digestion medium containing DNase I and protease XIV. During the incubation of minced endometrial tissue in method 1, the minced tissue aggregated due to mucus, which hindered cell isolation. Although DNase I was used in method 2 to break down DNA, the isolated cell suspension still appeared slimy, suggesting that loose DNA from dead cells is not the primary

cause of mucus formation. Furthermore, a study indicates that mucus-secreting goblet cells are particularly sensitive to dissociating enzymes (31). These cells may have died during the digestion period and released stored mucus.

To improve the isolation of primary epithelial cells with high cell density and cell viability, alternative methods should be considered. One option is to add Mucinase StcE, a metalloprotease that digests densely O-glycosylated mucins, to degrade mucus (32). However, live goblet cells might not be isolated since the dissociating enzymes could induce goblet cell death.

Additionally, a protocol for isolating mouse cervical epithelial cells using both protease and DNase I with a shorter incubation of 1h, achieved 90% cell viability, suggesting that the longer incubation time in method 2 may have negatively affected cell viability (31). This is further supported by comparisons of cell viability between uterine horn samples isolated using Method 1 and Method 2 demonstrated higher viability in Method 1.

Moreover, a study on human cervical epithelial cell isolation found that using dispase II combined with 0.25% Trypsin–0.01% EDTA for 20 hours significantly increased both cell density and viability, compared with isolation using collagenase I (33). Therefore, this enzyme combination could be considered for future isolations.

4.3 Primary porcine cervical epithelial cell condition

Due to time and material constraints, swine cervix cells were used for ALI culture. Since *C. fetus* has never been found in pigs, porcine cervical epithelial cells are not an optimal model to study adherence and invasion of *C. fetus*. However, the protocol for developing an ALI culture can still be established with these cell types, as porcine cervical epithelial cells grew well and differentiated. Although cultured porcine cervical epithelial cells detached from the edges of the inserts, the cells in the middle of the insert looked healthy. This suggests that the detachment may be due to one or more expired components in the medium that were no longer effective.

Cell differentiation was observed, with ciliated cells showing active cilia movement under the microscope, confirmed by BTUB staining. Unfortunately, mucus production was not detected, indicating the absence of goblet cells. This might be improved by adding oestrogens, which are mainly involved in the production of cervical mucus. Rapp *et al.* showed that gene expression related to mucus production in endocervical cells is regulated by hormone levels (34).

Studies using ALI cultures of porcine cervical, or oviduct epithelial cells did show mucus production without the addition of sex hormones (30,35). It is possible that the cervix of a 10-week-old pig is not yet capable of producing mucus, as this process occurred after premature (36). To verify this, mucin gene expression analysis could be performed. Another factor may be the use of LHC medium for the ALI culture, which is commonly used for bronchial epithelial cells. In contrast, the aforementioned studies using ALI cultures of porcine cells used HAM/F-12 (30,35). A review about ALI indicates that mucus production generally starts

after 2 weeks in ALI, while cultured porcine cervix epithelial cells were detached at the edge of the inserts after one week in ALI and did not have the chance to fully differentiate (22).

4.4 Bacterial staining

BacLight labelled *C. fetus* did not exhibit a clear green signal under the fluorescent microscope. A control should be included in the test to know whether mistakes were made during this experiment or if the labelling of C. fetus is ineffective. Furthermore, the exact molecule of BacLight remains unclear, it is known only as a non-nucleic acid labelling reagent, which raises concerns about its effect on bacterial cell activity. Therefore, BacLight bacterial staining is not an optimal labelling method for *C. fetus* cells.

Both CFSE and FISH successfully label *C. fetus*, though CFSE appears to be less optimal. CFSE signals were undetectable after overnight incubation, whereas FISH signals could still be detected. Additionally, some *C. fetus* cells were not visible with CFSE staining after 1 hour of incubation with epithelial cells but were detectable with DAPI staining.

Since most dead *C. fetus* cells should have been washed away during the fixation process. One possible explanation for the loss of CFSE signals could be rapid cell division during the 1h incubation with epithelial cells. However, studies show that *C. jejuni* has an average generation time of 2,2 hours in BHI (37). Since *C. fetus* grows slower than *C. jejuni*, and CFSE fluorescence was not visualised after 7-8 cell divisions, it raises the question of whether all *C. fetus* cells were properly stained. To clarify this, plate counting of the bacteria suspension could help confirm the actual cell numbers. In conclusion, FISH is the preferred labelling method for *C. fetus*, by preference using a *C. fetus*-specific probe to avoid labelling potential bacterial contaminants (38).

4.5 Infection assay

Results of the infection assay indicate that Cff adheres to porcine cervical epithelial cells with low adhesion ability, which is expected as pigs are not the natural host for *C. fetus*. *C. fetus* was observed primarily attaching to the cilia of the ciliated cells, which suggests that Cff may bind to specific receptors located on these cilia. Additionally, one *C. fetus* cell was found between or beneath the epithelial cell layers, indicating a potential ability to invade porcine cervical epithelial cells. However, since the edges of the cultured cells had detached from the inserts, it is unclear whether the bacteria penetrated the basolateral side through these gaps or actively invaded the cells. Further experiments with intact cell layers are necessary to clarify this.

C. fetus appeared to survive better in BHI medium than in DPBS-/-, this is likely due to DPBS-/- lacking amino acids and citric acid cycle intermediates that Cff can use as energy sources. This suggests that porcine cervical epithelial may produce insufficient components required for Cff survival. Furthermore, Gunther IV *et al.* demonstrated that small amounts of phosphates can influence the pH of the environment, resulting in reduced survival of *Campylobacter* cells (39). These could partly explain the lower adherence of *C. fetus* cells found in samples incubated in DPBS-/-, which contains a small amount of phosphates, although the results from Gunther IV *et al.* were based on *C. coli* and *C. jejuni (39)*. To explore whether these factors

contribute to the reduced attachment of Cff, future experiments could involve incubating *C. fetus* in HEPES-buffered medium to assess its nutrient requirements during infection.

The absence of amino acids or nutrients needed for bacterial survival could explain why few *C. fetus* cells remained after overnight incubation, as *C. fetus* need to obtain the components from the nutrient-rich BHI medium and may have consumed all available nutrients in BHI. This also explains why fewer *C. fetus* cells adhered to the epithelial cells at a higher MOI 500 compared to MOI 50, possibly due to competition for limited nutrients in the medium. This suggestion could explain why pigs are not the natural host for *C. fetus*, but other factors related to receptor-ligand binding could also play a role.

Cfv was not included in the infection assay due to limited materials cell inserts. The focus of this infection assay is on optimizing the model with the primary host cells by comparing different incubation conditions. Since neither Cfv nor Cff infects pigs naturally, studying their adhesion in this context may not be directly relevant. However, it would still be interesting to investigate whether Cfv can adhere to cervical epithelial cells, and if so, to determine if they have the same binding location as Cff, offering a deeper understanding of the adhesion mechanisms.

4.6 Virulence factors

SNPs in the *cdt* genes were identified in the genomes of Cff strains, with specific types of SNPs clustering together. Since strains within these clusters, sharing the same SNPs, originated from diverse hosts, it suggests that these SNPs are not associated with host preference. It is possible that these amino acid substitutions do not alter the binding sites of the Cdt proteins and therefore do not affect their function. If this is the case, the clustering of different strains may be driven by other nearby genes on the same locus rather than the *cdt* genes themselves. To determine whether these SNPs affect the function of Cdt, further structural analysis of the Cdt proteins, especially CdtA and CdtC, which are involved in host cell binding, is required.

Since all cfv strains consist of *sapA* genes, SapA may contribute to the niche preference of *C. fetus*. However, Cff strains containing SapA have been isolated from multiple species and Cff strains containing SapB, have also been found in cattle. This suggests that SapA may not play a role in host specificity. SapA could play a role in tissue tropism, this could be investigated by comparing the adherence and invasion of Cff strains from serotypes A, B, and AB on both intestinal epithelial cells and uterine epithelial cells.

Results of CaZyme analysis showed no differences in the types of CAZymes between the two *C. fetus* subspecies, except for variations in the copy numbers of some CAZymes. Cfv strains have higher copy numbers than cff strains, likely due to the larger genome size of Cfv. In addition, the analysis indicated that *C. fetus* has a limited capability to degrade mucin O-glycans. This suggests that other bacteria present in the cervix or intestine degrade mucins, thereby creating an opportunity for *C. fetus* to colonize the cervix and intestines. However, it is also possible that Cfv initially infects the uterus through direct contact with contaminated semen, subsequently spreading to the cervix of the cow, and thus does not need to require the ability to degrade mucins for infection.

4.7 Future research

In future studies, culturing bovine and ovine uterine epithelial cells in ALI with optimal setup will enable researchers to investigate the niche preference of *C. fetus* subspecies by comparing their adhesion and invasion on both cell types. It is expected that Cff will adhere and invade both bovine and ovine uterine epithelial cells, while Cfv is expected to only adhere and invade the bovine uterine epithelial cells. The results from this comparison could provide insight into whether host specificity is influenced primarily by factors affecting adhesion or invasion.

Graham and his colleagues demonstrated that the extracellular matrix (ECM) plays a role in Cff adhesion to INT 407 cells, with *C. fetus* binding to immobilized fibronectin and using soluble fibronectin to enhance attachment to other ECM components (13). This suggests that the ECM may also influence *C. fetus* niche preference. To further investigate this *in vitro*, a possible follow-up study could include creating a 3D scaffold-based model, which could offer a more physiologically relevant environment than the ALI model. This model is an improved version of the ALI culture, in which stromal cells were cultured on the underside of the porous inserts while keeping the rest of the setup the same. The stromal cells would produce ECM, resulting in the incorporation of cell-cell and cell-ECM interactions, thus simulating a real microenvironment for *C. fetus* infection (40)

Bücker *et al.* demonstrated that *C. fetus* can translocate both paracellularly and transcellularly in HT-29/B6 cells. However, paradoxically, a study by Baker and Graham showed that *C. fetus* only translocated transcellular in Caco-2 cells (15,17). The proposed 3D scaffold-based model, which simulates a more realistic microenvironment for *C. fetus* infection, could help further investigate and clarify the translocation pathway of *C. fetus*. This model would also allow for a more accurate assessment of *C. fetus*'s interaction with the ECM and host cells.

4.8 Conclusion

With this study we found that Cff adheres to porcine cervical epithelial cells, demonstrating that the ALI model is an effective model for studying the niche preference of *C. fetus*. However, the current setup requires further optimization, particularly concerning the isolation methods for bovine uterine epithelial cells, which remain the most challenging aspect. Refining these methods is essential for optimizing the model.

Despite this, the ALI culture of bovine epithelial cells, following the protocol used for porcine cervical epithelial cells, could still be used since cell differentiation has been successfully achieved. For further experiments, it is recommended to use *C. fetus* in the log phase, cultured in BHI, to infect epithelial cells. The infection conditions should involve a MOI of 50 and 1-hour incubation in BHI. Finally, using FISH as a labelling technique is preferred as it is an effective marker for detecting bacterial cells.

Future research into culturing bovine and ovine uterine epithelial cells in ALI with optimal setup could facilitate the investigation of the niche preference of *C. fetus* subspecies by comparing their adhesion and invasion on both cell types. This approach could provide insight into the virulence mechanisms underlying infection of *C. fetus* and its hosts.

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7. Appendix

7.1 *C. fetus* genomes

Table 1. Characterization of 50 C. fetus genomes used for genome analysis

Genomes	Host	Country	Subspecies	Genome size (BP)
2160129049	Equine	NL	Cff	1833854
04554	Bovine	Argentina	Cff	1826623
15S04041-1	Human	NL	Cff	1762383
15S04042-1	Human	NL	Cff	1828464
15S04240-1	Human	NL	Cff	1762758
15S04243-1	Human	NL	Cff	1741961
16S00675-10	Equine	NL	Cff	1756794
16S05798	Human	NL	Cff	1783784
17S01351-1	Ovine	NL	Cff	1803718
17S01353-1	Ovine	NL	Cff	1745259
17S03759-1	Human	Germany	Cff	1841202
18S02845-1	Unknown	US	Cfv	1895752
18S02849-1	Bovine	US	Cfv	1854416
18S02853-1	Bovine	US	Cfv	1801219
18S02857-1	Bovine	US	Cff	1727602
18S02858	Bovine	US	Cfv	1953718
18S02863	Ovine	US	Cff	1755235
18S03233-1	Bovine	US	Cfv	1930028
18S03801-1	Bovine	US	Cfv	1830165
18S03802-1	Unknown	US	Cff	1748960
18S03803-1	Unknown	US	Cfv	1885636
18S03806-1	Unknown	US	Cff	1736360
18S03808-1	Unknown	US	Cff	1729520
18S03823-1	Ovine	US	Cff	1724540
18S03824-1	Ovine	US	Cff	1754192
18S03831-1	Bovine	US	Cfv	1978883
18S03841-1	Bovine	US	Cff	1743099
18S04140-1	Human	Belgium	Cff	1744392
19S00791-1	Human	India	Cff	1782846
19S00906-1	Bovine	US	Cfv	1856846
925	Bovine	Argentina	Cff	1828367
B0042	Bovine	UK	Cff	1801309
83	Bovine	New-Caledonia	Cfv	2071080
Ru-2009-08871-5 direct	Bovine	NL	Cfv	1982467
78-1	Human	US	Cff	1743581
82-40	Human	US	Cff	1773615
84-112	Bovine	Argentina	Cfv	1988028
ERR1046006	Human	Taiwan	Cff	1769351
ERR1046007	Human	Taiwan	Cff	1785728
ERR1046008	Human	Taiwan	Cff	1800610
ERR1046010	Human	Taiwan	Cff	1884758

ERR1046013	Human	Taiwan	Cff	1778253
ERR1046021	Human	Taiwan	Cff	1790092
ERR1069017	Bovine	Germany	Cff	1795127
ERR1069024	Bovine	Germany	Cff	1792875
ERR1069029	Ovine	Germany	Cff	1748080
ERR976358	Human	France	Cff	1796562
ERR976364	Human	France	Cff	1818678
NCTC10354	Bovine	UK	Cfv	1858789
NCTC10842	Ovine	France	Cff	1763253

Cfv = *Campylobacter fetus venerealis*; Cff = *Campylobacter fetus fetus*; UK = United Kingdom; US = United States; NL = The Netherlands; BP = base pair

7.2 Codes used in Linux

Genome size

1. cat 82-40.fasta |grep -v ">" |tr -d "\n" | wc -c
2. for genome in *.fasta; do size=$(cat "$genome" |grep -v ">" |tr -d "\n" | wc -c); echo "$genome: $size"; done

Phylogenetic trees using parsnp

1. cd ~/cfetus_test/
2. parsnp -c -d cleanedup/ -r ! -o output_parsnp

Annotation using bakta

1. cd ~/cfetus_test/
2. source /usr/local/anaconda/etc/profile.d/conda.sh
3. ~/cfetus_test$ conda activate
4. bakta --db /mnt/DGK_KLIF/data/db/bakta/db --output bakta/ --genus Campylobacter file name.fasta
5. ls -1 |grep fasta$|cut -f 1 -d . |while read genome ; do bakta --db /mnt/DGK_KLIF/data/db/bakta/db --output bakta/$genome --genus Campylobacter $genome.fasta ; done

BLASTp for *cdt* genes

1. cat cdtA.faa cdtB.faa cdtC.faa > cdt.faa
2. cd cdt_protein_*C. fetus*/
3. makeblastdb -in cdt.faa -dbtype prot -hash_index
4. for faa in */*.faa; do blastp -db ~/cfetus_test/cdt_protein_*C. fetus*/cdt.faa -outfmt "6 qseqid sseqid qlen slen qstart qend sstart send length pident positive mismatch gaps evalue bitscore qcovs qseq sseq" -query "$faa" | while read -r hit; do echo "$faa $hit"; done; done > cdt_aa5.out
5. echo -e 'Strain\tqseqid\tsseqid\tqlen\tslen\tqstart\tqend\tsstart\tsend\tlength\tpident\tpositive\tmismatch\tgaps\tevalue\tbitscore\tqcovs\tqseq\tsseq' > blastp_header2.tab
6. cat blastp_header2.tab cdt_aa5.out > cdt_aa5.tsv
7. column -t cdt_aa5.tsv | head -20
8. sed 's/\t/\n/g' blastp_header2.tab | cat -n
9. awk 'BEGIN{FS=OFS="\t"} $10 >= 95 && $16 >= 97' cdt_aa5.tsv | column -t
10. awk 'BEGIN{FS=OFS="\t"} $10 >= 95 && $16 >= 97' cdt_aa5.tsv | column -t > ctdgene_with_seq.list
11. awk 'BEGIN{FS=OFS="\t"} $10 >= 95 && $12 >=1 && $16 >= 97' cdt_aa5.tsv | column -t > cdtgene_mismatch.list

Blastn for *sap* genes

1. nano sap_primer.fasta
2. makeblastdb -in sap_primer.fasta -dbtype nucl -hash_index
3. for fna in */*.fna; do blastn -task blastn -dust no -soft_masking false -db ~/cfetus_test/sap_primer.fasta -outfmt "6 qseqid sseqid qlen slen qstart qend sstart send length pident positive mismatch gaps evalue bitscore qcovs qseq sseq" -query "$fna" | while read -r hit; do echo "$fna $hit"; done; done > sapgene2.out
4. cat ../sap_header2.tab sapgene2.out > sapgene2.tsv
5. awk 'BEGIN{FS=OFS="\t"} $10 >= 95 && $9 >= 18' sapgene2.tsv | column -t > sapgene.list

7.3 Growth curves of *C. fetus* in DMEM

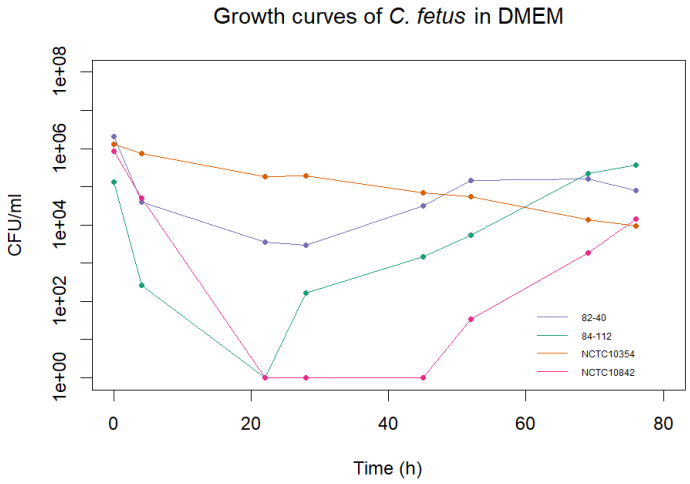

Figure 1. Growth curves of the four laboratory C. fetus strains grown in DMEM at 37◦C, 160 rpm for 76h*. Inoculations were made with 100 µl of 0,5 McFarland and measured at 0h, 4h, 22h, 28h, 45h, 52h, 69h and 76h using plate counting.*

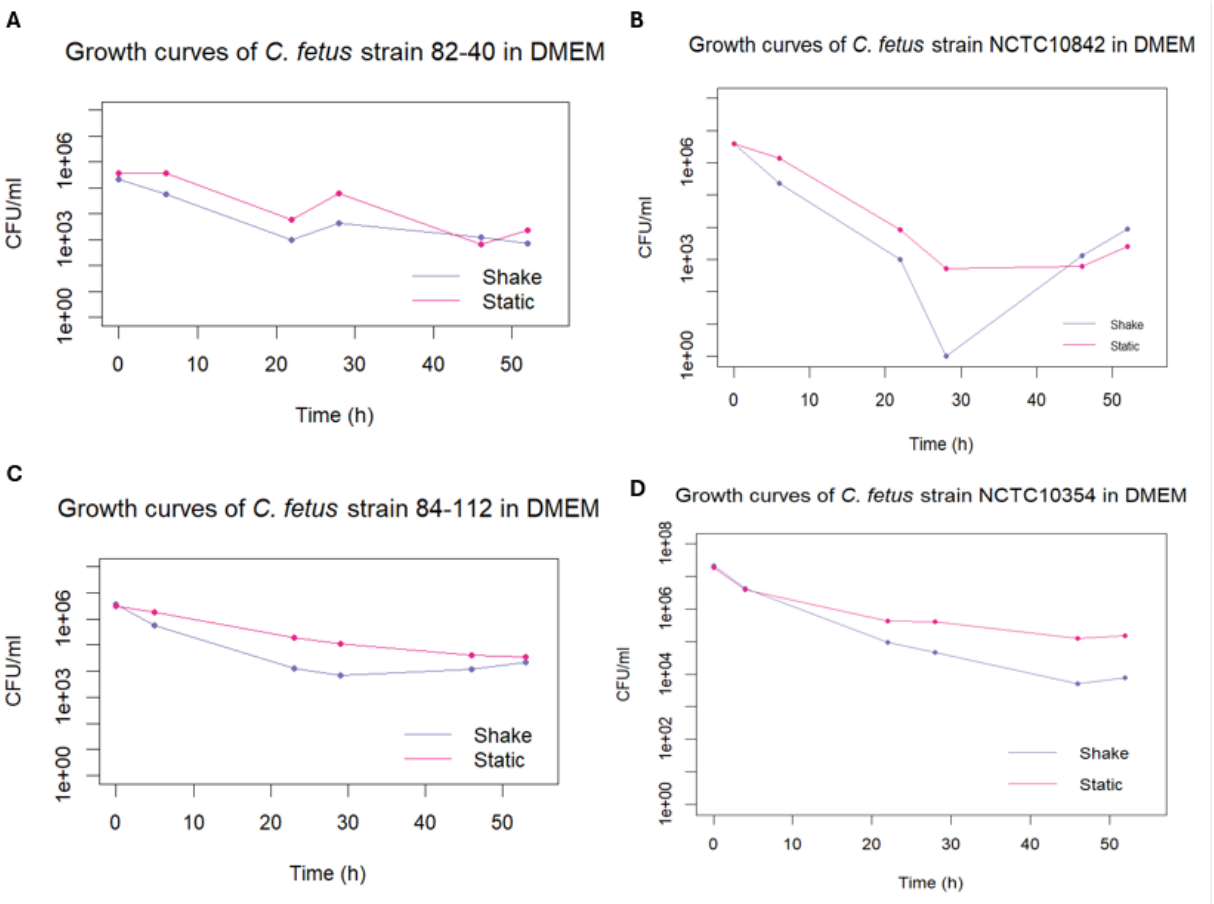

Figure 2. Growth curves of the four laboratory C. fetus strains cultured in DMEM under shaking or static conditions at 37◦C and 160 rpm for 52h or 53h. *A: Growth curves of Cff strain 82-40 in DMEM; B: Growth curves of Cff strain NCTC10842 in DMEM. C: Growth curves of Cfv strain 84-112 in DMEM; D: Growth curves of Cfv strain NCTC10354 in DMEM. The growth of C. fetus in DMEM was analysed by plate counting. For Cff strain, measurements were taken at 0h, 6h, 22h, 28h, 45h, and 52h, while for Cfv, they were taken at 0h, 5h, 23h, 29h, 46h, and 53h.*

7.4 SNP analysis of *cdt* genes

Table 2. 50 C. fetus genomes and their SNPs found in cdt genes

***C. fetus* genomes**	***cdtA* gene**	***cdtB* gene**	***cdtC* gene**
04554	p.L77P	p.V168I and p.A209T	
15S04041-1	p.H91Y	p.M6I	
15S04042-1			p.I6T and p.P58S
15S04240-1	p.H91Y	p.M6I	
15S04243-1		p.M6I	
16S05798			p.S67A
16S00675	p.V267A		
17S01351-1	p.H91Y	p.M6I	
17S01353-1			p.I6T and p.P58S
17S03759-1			p.I6T and p.P58S
18S02845-1			
18S02849-1			
18S02853-1			
18S02857-1			
18S02858			
18S02863	p.H91Y	p.M6I	
18S03233-1			
18S03801-1			
18S03802-1			p.I6T and p.P58S
18S03803-1			
18S03806-1			p.I6T and p.P58S
18S03808-1			p.I6T and p.P58S
18S03823-1		p.M6I	p.S67A
18S03824-1	p.H91Y	p.M6I	
18S03831-1			
18S03841	p.V267A		
18S04140	p.V267A		
19S00791-1		p.M6I	p.S67A
19S00906-1			
2160129049	p.D49E	p.V168I and p.A209T	
24S00315-1	p.H91Y	p.M6I	
24S00316-1	p.L77P and p.G115D	p.V168I and p.A209T	
24S00317-1			
24S00318-1			
78-1			p.I6T and p.P58S
82-40	p.V267A		
84-112			
ERR976358	p.V267A		
ERR976384	p.V267A		
ERR1046006	p.V267A	p.M6I	p.S67A
ERR1046007	p.V267A	p.M6I	p.S67A
ERR1046008			p.S67A
ERR1046010			p.S67A
ERR1046013	p.V267A	p.M6I	p.S67A

ERR1046021			p.S67A
ERR1069024			p.I6T and p.P58S
ERR1069029			p.I6T and p.P58S
ERR1069017	p.V267A		
NCTC10354			
NCTC10842			p.I6T and p.P58S

7.5 CAZyme types

Table 3: Types of CAZyme found in 8 C. fetus genomes

C. fetus	GH	CE	GT	CBM	AA
	GH23	CE11	GT2		AA1
	GH73	CE4	GT51		AA4
	GH3		GT30		
			GT66		
			GT4		
			GT9		
			GT19		
			GT28		
Total	3	2	8	0	2

GH = glycoside hydrolases, CE = carbohydrate esterases, GT = glycosyl transferases, CBM = carbohydrate-binding modules, AA = auxiliary activities

7.6 Cell viability of isolated bovine epithelial cells

Table 4. Isolated bovine epithelial cells and their cell viability.

Cell origin	**Concentration cell suspension** (cells/ml)	**Cell viability**(%)	**Total cell suspension** (ml)
Horn cow 1 (1)	0,5 M cells/ ml	93%	9 ml
Horn cow 2 (1)	0,5 M cells/ ml	70%	6 ml
Horn cow 1 (2)	NA	NA	NA
Horn cow 2 (2)	1 M cells/ ml	11%	8 ml
Cervix cow 1 (2)	1,18 M cells/ ml	44%	8 ml
Cervix cow 2 (2)	0,6 M cells/ ml	20%	8 ml
Placenta cow 2 (2)	2 M cells/ ml	29%	12 ml
Fetal trachea cow 2 (2)	0,85 M cells/ ml	94%	8 ml

(1): isolation method 1; (2): isolation method 2; NA = not applicable