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Effects of the naturally occurring inhabitants of the vaginal microbiota of cows on cultured endometrial cells

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List of Abbreviations:

AGP= Antimicrobial Growth Promoters

AMR= Antimicrobial Resistance

APM= Acute Puerperal Metritis

B = Bacillus

Bb = Brevibacillus

BEnEpC = Bovine Endometrial Epithelial Cells

CR= Conception Rate

DIM= Days in Milk

E. coli = Escherichia Coli

ESBL = Extended Spectrum β-Lactamase

F = Forward primer

FBS= Fetal Bovine Serum

HI= Heat- Inactivated

IUPEC = Isolated Intrauterine Pathogenic Escherichia Coli Strains

LAB= Lactobacillus

MLST= Multi-Locus Sequence Typing

MOI= Multiplicity of Infection

NEFA = Non- Esterified Fatty Acids

NRR=Non-Return Rate

NSAIDs = Non-Steroidal Anti-Inflammatory Drugs

OTU= Operational Taxonomic Unit

PMNs = Polymorphonuclear Neutrophils

R = Reverse primer

RAPD =Random Amplification of Polymorphic DNA

T = Trueperella

WHO= World Health Organization

1 Introduction

Cattle reproduction is often less effective than that of other livestock animals with a limit of one pregnancy per year. For that reason, finding bovine reproductive indicators that can forecast high pregnancy possibility is a key for enhancing bovine reproductive efficiency (Ong et al., 2021).

One of the main causes of the dairy industry's significant financial losses is bovine subfertility. Uterine alterations in the postpartum period might be connected to poor reproductive ability, such as extended intervals between calving and conception (Peter et al., 2018).

It was widely accepted for a long time that animals' female reproductive tracts were sterile organs. Microbes have historically been detected using culture dependent methods. While changes of the microflora of the reproductive system are likely to have a detrimental influence on health, molecular approaches imply that not all bacteria in the microbiota indicate sickness (Appiah et al., 2020).

Despite the fact that a lot of typical reproductive issues in animals are caused by bacterial infection, very little is known about their typical vaginal microbiome (Swartz et al., 2014).

Understanding the microbial colonies that reside in cow's reproductive canal may help us comprehend the physiology of the animal's reproductive tract, and that is of huge commercial value (Laguardia-Nascimento et al., 2015).

The objective of reproduction regulation is to get cows conceived at a period that is both physiologically and economically advantageous (Sheldon et al., 2006). Before the next parturition, uterine involution, endometrial regeneration, a return to ovarian cyclic function, and the management of harmful microorganisms in the uterus are all necessary (Sheldon and Owens, 2017). Nonetheless, the natural involution process should take place, providing enough time for therapy and reaction before breeding begins (Sheldon et al., 2006).

2 Literature review

2.1 Normal pH of the vagina and uterus

PH is considered one of the main intrinsic defensive systems of the body as it prevents pathogens through skin, digestive tract and mucus membrane (Elad and Beckwith-Cohen, n.d.).

The Henderson-Hasselbalch equation, which describes pH in its most basic form as the negative logarithmic measure of the hydrogen ions contained in a solution (Roper, 2014).

The pH of blood and the body as a whole it is 7.4 and this value has long been considered as the baseline for homeostasis. Nonetheless, this is not always the case from a reproductive perspective, especially when considering the uterine or the vaginal environment (Roper, 2014).

Based on a study finding (Inas et al., 2017), sixty female dairy cows were applied. At the time of artificial insemination, a digital pH meter is used to assess the vaginal mucosa on each dairy cow. The findings may well be established that the vaginal mucosa's pH 7.2 is the ideal pH for achieving the maximum rate of conception. The findings were excellent, and the percentage of Conception Rate (CR) is 88.33% and non-return rate (NRR) is 86.79% (Inas et al., 2017). On the other hand according to Beckwith-Cohen (2012), beginning with a median pH value of 7.25, first-calf heifers rapidly became more alkaline within the first week after delivery, reaching a median pH value of 7.75, and subsequently dropped to an average value of 7.5 (Elad and Beckwith-Cohen, n.d.).

However, when assessing pH levels in a particular cattle population during the periparturient phase, the difference between cows and first calf heifers was noteworthy. When examining the influence of days in milk (DIM) on pH values for the general cattle population, no important variations were observed (Elad and Beckwith-Cohen, n.d.).

2.2 Normal microbiota of the uterus

The word 'microbiota' refers to a variety of microorganisms that naturally inhabit in a specific environment.

However, when these colonies are disturbed, it can negatively impact physiological processes (Appiah et al., 2020). Comprehension of the microbial communities that live in the cow's vaginal canal may allow us to understand the physiology of the animal and its reproductive system, which is of enormous commercial significance (Laguardia-Nascimento et al., 2015). In the healthy microbiota of the bovine urogenital tract, strict anaerobic, facultative anaerobic, and aerobic microbes coexist in a dynamic mix (Wang et al., 2013). In an environment that is vibrant and active, new strains are constantly being introduced. *Staphylococcus* and *Streptococcus* bacteria make up the majority of the usual microbiota in this tract (Otero et al., 2000).

During the beginning delivery, the physical cervical barrier is damaged, making it possible for germs to enter the genital system through the vagina from the vaginal area or the surroundings, as well as from animal skin and excrement (Appiah et al., 2020).

Some potential sources of the microbiota for the reproductive tract are the blood, from the environment and directly from the vagina (Appiah et al., 2020).

A culture independent method has been used to examine the vaginal microbiota in Nellore cattle breed; heifers and cows; pregnant and non-pregnant. *Firmicutes* were found in 40%-50% of the bacteria, followed by *Bacteroidetes* (15%-25%) and lastly *Proteobacteria* (5%-25%). In addition, 10-20% of unclassified bacteria were found. Only ten OTUs; *Aeribacillus, Bacteroides, Clostridium, Ruminococcus, Rikenella, Alistipes, Bacillus, Eubacterium, Pevotella* and non-classified bacteria constituted 45%-55% of the samples (Laguardia-Nascimento et al., 2015).

In a study where ten series of 15 vaginal samples were collected through an 18- month period, while maintaining 10^2-10^4 CFU/ml sample, enterococci and staphylococci were the primary microorganisms, with enterococci having a somewhat greater frequency than staphylococci. A smaller number of bacteria were found in the *Enterobacteriaceae* and lactobacilli groups (Otero et al., 2000).

Comparing bacteria isolated from the vagina of healthy pregnant and diseased postpartum cows, it has been discovered that both healthy and diseased cows included populations of bacilli and lactic acid bacteria from the genera *Enterococcus, Lactobacillus* and *Pediococcus*. The number of vaginal enteric bacteria, primarily *Escherichia coli*, significantly increased in infected cows (Wang et al., 2013).

Not only *Bacteria* were found in the vaginal microbiota of the Nellore cattle but also *Archaea* and fungi. *Methanobrevibacter* was found to be prevalent. The hormonal state of the host affected the abundance of this archeal genus as well. (Laguardia-Nascimento et al., 2015). This species plays a crucial role in the metabolism of plant compounds (Hristov et al., 2012).These findings show a strong relationship between the vaginal and GIT microbiota in cattle. The existence of fungus was the main focus of the eukaryote study, predominantly *Ascomycota* with a tendency to reduce pregnancy state (Laguardia-Nascimento et al., 2015).

2.3 Pathological alterations

Despite the unquestionable negative effects on reproductive outcomes, uterine illnesses' cause decreased productivity and reduced fertility (Sheldon and Owens, 2017). All cows have a contaminated uterus following calving (Pulfer and Riese, n.d.). This is usually eliminated from the cows within a few weeks. Sometimes, this contamination is not completely removed in a small percentage of cows, and the uterus develops an infection ("NADIS Animal Health Skills - Fertility in dairy herds," n.d.). Pathogenic bacteria, including *Escherichia coli, Trueperella pyogenes, Fusobacterium necrophorum, Prevotella and Bacteroide*s, are linked to postpartum uterine illness (Sheldon and Owens, 2017). In fact *T. pyogenes, F. necrophorum* and *Prevotella* work cooperatively and increase the severity of endometritis (Sheldon and Owens, 2017).

Nevertheless, according to culture-dependent study findings *Trueperella pyogenes* is a key pathogen engaged in the creation of clinical endometritis and that other Gram-negative anaerobes, including *Fusobacterium necrophorum, Porphyromonas levii, and Prevotella melaninogenica*, may work in concert with *T. pyogenes* to cause clinical endometritis (Galvão et al., 2019).

Furthermore, studies also support the role of *Escherichia coli*, either as the main pathogen or as a secondary pathogen that opens the path for *T. pyogenes* and Gram-negative anaerobes. *T. pyogenes, E. coli* and Gram-negative anaerobes appeared to be implicated in the development

of metritis, even if there was just a lack of data for cows with metritis (Galvão et al., 2019). Around 2010, researchers have used Random Amplification of Polymorphic DNA (RAPD) and Multilocus Sequence Typing (MLST) to study the microbiome of cows with metritis. Distinct clonal groups of E. coli were found in animals with uterine illness that were different from recognized diarrhoeic or extra-intestinal E. coli. When compared to E. coli isolated from the uterus of clinically unaffected individuals, the endometrial pathogenic E. coli (EnPEC) were more adherent and invasive for endometrial epithelial and stromal cells (Sheldon et al., 2010).
Although alterations in the reproductive tract's microbiome are likely to have a negative impact on health, molecular techniques suggest that some abnormalities in the biota of the reproductive tract are relatively harmless and may not always indicate illness (Appiah et al., 2020).

2.4 Diseases of the Uterus

Uterine illness typically reveals itself during the 'postpartum phase' which is defined as the interval between giving birth and the termination of uterine involution (Sheldon and Owens, 2017). Up to 50% of all dairy cows experience postpartum uterine inflammation (Sheldon et al., 2009).
As reported by Sheldon and colleagues, metritis and endometritis are the two most common postpartum clinical disorders in cows (Sheldon and Owens, 2017).
The term 'metritis' describes a bacterial infection of the entire uterus. It is most prevalent within 10 days following parturition (Sheldon and Owens, 2017). Although, this can range from modest (decrease in appetite and milk output) to extremely severe and perhaps lethal, it is always linked to systemic disease. Severe or puerperal metritis frequently develops in the first few days following calving and it is usually a complication of difficult aided calving that damaged the reproductive canal and introduced germs. ("NADIS Animal Health Skills - Fertility in dairy herds," n.d.). It is also defined by an enlarged uterus filled with a red-brown fluid to off-white discharge that frequently has a fetid smell (Sheldon and Owens, 2017).
Galvao and colleagues' recent research also shown a link between metritis and uterine dysbiosis, defined by elevated *Bacteroidetes* and *Fusobacteria* number (Galvão et al., 2019).
Another significant postpartum illness is thought to be acute puerperal metritis (APM). APM develops within 21 days of giving birth and is defined by fever, an inflated uterus and a watery red-brown fluid and purulent discharge (Haimerl et al., 2017; Haimerl and Heuwieser, 2014).

Reduced milk production, dullness or other toxemia symptoms, a drop in DMI, an increase in heart rate, and dehydration are all indicators of systemic disease (Sheldon et al., 2008).

However, Appiah and colleagues state that there appears to be a significant link between metritis and endometritis, and that any possibility of infection with metritis occurring just after delivery would indirectly increase the probability of endometritis developing (Appiah et al., 2020).

Endometritis is perhaps the most prevalent disease according to UK statistics. Endometritis has two major impacts on fertility. In the beginning, it is linked to lack of heat behavior, which delays ovulation after calving and prolongs the time between ovulations. In the second place, cows with endometritis are considerably less likely to conceive even while they are in their oestrus cycle. The reason is that the infected uterus is an inappropriate habitat for the growing embryo ("NADIS Animal Health Skills - Fertility in dairy herds," n.d.).

Endometritis was further divided into clinical and subclinical categories, with the clinical is identified by the appearance of discharge while the subclinical is defined by inflammation of the endometrium without any clinical illness (Lima, 2020).

Pyometra is connected to ovarian corpus luteum activity and frequently lasts longer than the anticipated luteal phase length. The uterine canal fills up with fluid and produces mucopurulent discharge and a closed cervix (Rosales and Ametaj, 2021). It has been hypothesized that the existence of this structure, together with the progesterone it secrets, is what causes endometritis to progress to pyometra (Sheldon et al., 2006).

2.5 Treatment possibilities

Treatments for uterine diseases attempt to improve uterine defense and repair while reversing inflammatory alterations that reduce fertility (Sheldon et al., 2006).

Even though treating metritis that is not showing systemic signs is not a medical emergency, it is crucial for the animal's ability to reproduce in the future. Treatment for metritis should aim to get rid of the infection of the uterus with the least amount of tissue damage possible. This makes it possible to conceive quickly following therapy (Pulfer and Riese, n.d.). Three categories of treatments are often used (Table 1): antibiotic therapy, chemical disinfectants and hormone therapy (Pulfer and Riese, n.d.; Rosales and Ametaj, 2021).

Antibiotics

While only neomycin is approved for use during cow intrauterine treatment, numerous additional other antibiotics, including penicillins, tetracyclines, aminoglycosides, nitrofurazone and sulfonamides have been utilized for extra-label use (Pulfer and Riese, n.d.)

Although sulfonamides' efficacy against the pathogenic organisms looks good *in vitro*, as they prevent the production of bacterial folic acid, sulfonamides can also be extremely irritating to the endometrium (Pulfer and Riese, n.d.).

Aminoglycosides also exhibit a wide range of action *in vitro*, but bacteria must have access to oxygen for oxidative phosphorylation to take them up. Therefore, in the low oxygen environment of the diseased uterus, their effectiveness is greatly reduced (Olson et al., 1984).

In its regularly used concentrations, nitrofurazone has trouble reaching the minimal inhibitory concentration required for bacterial inhibition (Olson et al., 1984).

Penicillin appears to be less irritating to the endometrium nevertheless the diverse bacterial flora of the early postpartum uterus makes it a poor option due to its bacterial resistance (Pulfer and Riese, n.d.).

APM was mostly treated with one antibiotic, (ceftiofur), in 17 out of 23 investigations, which is significant and may be regarded as extensively investigated. The effectiveness of this antibiotic was shown in 13 (7 studies showed improvement) and 6 (0 studies showed improvement) investigations, respectively, with regard to clinical and reproductive characteristics (Haimerl and Heuwieser, 2014).

Recent research by Oliveira and others demonstrated that giving ceftiofur to cows with metritis enhanced cure rates but did not reduce culling in the first 60 days after delivery. Instead, it boosted milk production and fertility up to 300 days after delivery (Oliveira et al., 2020).

Contrarily, ceftiofur usage has been linked to significant alterations in the phylum and genus levels of microbiota, compared to ampicillin usage, which had no impact on uterine microbiota (Jeon et al., 2018; Rosales and Ametaj, 2021).

Disinfectants

For the treatment of metritis, intrauterine administration of disinfectants such as iodine, or chlorhexidine has been recommended. Heavy iodine solutions, according to studies, might result in necrosis of the endometrium and a return to estrus in 4-29 days. It has been shown that the

disinfectant's irritation causes a prostaglandin release, which leads to luteolysis and oestrus return. (Pulfer and Riese, n.d.).

Hormone therapy

Utilizing hormones such as oxytocin, ergonovine, estrogens and prostaglandins will encourage uterine evacuation and strengthen uterine protection. Immediately after delivery, oxytocin is frequently given to promote placenta evacuation and induce uterine contractions. Ergonovine is a product of a fugus, not a hormone. Controlled studies have indicated that contraction of the myometrium is limited or unaffected. However, oestrogen is the only medicinal substance that causes uterine contractions at all phases of the cycle, and when administered before day 40 postpartum, it has been demonstrated to be at least as good as prostaglandins (Pulfer and Riese, n.d.).

Uterine Infection	Antibiotics	Hormones	Disinfectants
Metritis, endometritis, and pyometra	Penicillin,ampicillin,ceftiofur, oxytetracycline, cloxacillin, aminoglycosides,nitrofurazone and sulfonamides + Distilled water or saline	1.Prostaglandins and Estrogens 2.Oxytocin and ergonovine	Iodine,chlorhexidine or cresol
Application	Infusion and injection	Injection	Infusion
Disadvantages	-Microbial resistance - Residues -production cost increase -Endometrial irritation	- Production cost increase -Contraction of the uterus regarding the size of the cervix	-Endometrial necrosis and irritation of epithelium

Table 1. Treatment of postpartum uterine infections in dairy cows by Rosales and Ametaj, 2021

There have been several research that have investigated additional non- conventional therapies against uterine infections in addition to the usual treatments discussed above. Non-steroidal anti-inflammatory medicines (NSAIDs) were utilized in several of these trials during the first postpartum week. These research' findings have been inconsistent. According to one study, milk output was positively impacted contrary to glucose and non- esterified fatty acids (NEFA) which were negatively impacted (Rosales and Ametaj, 2021).

2.6 Unnecessary use of Antibiotics/ Spread of Resistance

One of the biggest challenges to public health in the twenty-first century in already widely acknowledges to be antibiotic resistance (Haimerl and Heuwieser, 2014).

Numerous theories, including immunosuppression, periparturient diseases, and intrauterine insertion of chemical substances lowering the defensive mechanism of cows, have been put up explaining the poor cure rate with systematic antibiotics administration (Smith et al., 1998).

Additionally, it was hypothesized that the ongoing utilization of antibiotics in dairy farms contributed to the spread in antimicrobial resistance (AMR) , particularly to the widely used antibiotic regimens, which ultimately caused antibiotics to lose their efficacy (Ma et al., 2018).

AMR in producing animals is not only a veterinary issue as it is receiving a lot of attention because of its connection to human health as well (Appiah et al., 2020).

A study work which examined the frequency of extended spectrum β-lactamase (ESBL) producing bacteria in uterine samples from cows with metritis to describe the isolated intrauterine pathogenic *Escherichia coli* strains (IUPEC). As a result the metritic cows investigated, had a significant proportion of ESBL-producing IUPEC with multi-drug resistance, including ceftiofur, which is frequently used to treat metritis (Ma et al., 2018).

Furthermore, the high frequency of *E. coli* that is resistant to several different drugs, it is linked to bovine endometritis. The discovery of the *fimH* gene provides indirect evidence that this gene is essential for the development of biofilms in intrauterine pathogenic *E. coli* (Raheel et al., 2020).

2.7 Limitations of antimicrobial use in farm animals

As stated by the WHO (World Health Organization), the danger for the creation and transmission of bacteria resistance has increased due to the extensive use of antimicrobials in farm animals, both therapeutically and prophylactically as well as to promote growth. (Aidara-Kane, 2012; Manishimwe et al., 2017; McEwen and Collignon, 2018). Consequently, new rules have been released by WHO lately where farmers and the food industry are advised to avoid consistently administering antimicrobials for the exception of therapeutic use only (McEwen and Collignon, 2018).

According to a research conducted by Manishimwe and colleagues in 2017 44.4 and 26.5% of farmers in Rwanda claimed they administered antibiotics to avoid sickness and to promote growth, respectively (Manishimwe et al., 2017).

This matter is of critical importance as the resistance can be transmitted to humans by the ingestion of animal source food, close contact with the animal or environmental dispersal. Given that when both people and animals utilize the same kinds of antimicrobial medicines, this poses specific safety concerns (Aidara-Kane, 2012).

A recent study investigated the effect of the removal of the antimicrobial as growth promoters. Despite the Swedish prohibition on AGP (Antimicrobial Growth Promoters) in 1986, the number of preventive antimicrobials used in animals grew by 21% until 1988, didn't change until 1994, and then decreased by 47% by 2003. Pig population in Sweden decreased by 16% throughout this time, while other species' populations varied, with the exception of chickens, who saw a rise (McEwen et al., 2017).

When AGPs were eliminated in Norway in 1995, a 39% decline in therapeutic usage has been reported, between 1995 and 2000, after which it remained almost the same until 2003. Between 1995 and 2003 Norway's pig population expanded by roughly 10%, and there were significant rises in poultry output too (McEwen et al., 2017).

Although the evident expected result of the avoidance of antibiotics as suggested by WHO, a new study suggests that there might be unforeseen outcomes such as damage to animal health and rising production costs. These consequences are not inevitable. Revisions to animal health management and housing should be taken into consideration to lessen the risk for this matter (McEwen et al., 2018).

2.8 Probiotic bacteria and their benefits

Bovine uterine diseases decrease the cow's ability to reproduce, raise the expense of herd health, reduce feed intake, and lower milk output. Most of the treatments for those diseases contaminate milk. These circumstances compel farmers to cull cows (Otero et al., 2006).

Probiotics are an excellent alternative to antibacterial drugs due to their ability of competitive exclusion and immunomodulatory action. Consequently, the scientific community has suggested using probiotics as an alternative to antibiotics (Appiah et al., 2020).

Probiotics are living microorganisms that, when given to a host in sufficient quantities a positive impact on their health is observed, according to the Food and Agriculture Organization of the United Nations (FAO) (Rosales and Ametaj, 2021).

Probiotic bacteria have a wide range of additional ways to benefit both the host and themselves. They can benefit the host through a number of different mechanisms, some of which are as follows: (1) enhancing epithelial barrier functions, (2) creating biofilms on mucosal layers, (3) preventing pathogen adhesion, (4) competing for essential nutrients, (5) producing anti-microbial compounds, (6) modulating the immune system and (7) altering the vaginal pH (Bermudez-Brito et al., 2012).

In a study research several examples were provided of how intravaginal lactobacilli have reduced uterine infections, enhanced immune responses, and increased milk production (Rosales and Ametaj, 2021). While another study investigation demonstrated that a large number of different strains of *Lactobacillus sp*. and *Streptococcus sp*. suppressed vaginal bovine *Escherichia coli* in the agar plate diffusion test. Although, in this study, *Actinomyces pyogenes*, a pathogen identified from bovine metritis, was inhibited by only a few strains (Otero et al., 2006).

In contrast to other *Lactobacillus* species, it was demonstrated in an earlier investigation that *Lactobacillus buchneri* did not affect the viability of endometrial epithelial cells or cause an inflammatory response. Consequently, *L. buchneri* appears to be suitable to function as a probiotic strain (Peter et al., 2018).

Hundred lactating Holstein dairy cows were used to determine if periparturient uterine infections and general health status may be affected by intravaginal infusion of a lactic acid bacteria (LAB) cocktail. In fact, evidence indicated that cows treated with LAB had decreased incidences of metritis and overall uterine infections as well as enhanced innate immune

responses locally and systemically. Conversely, given the dose and frequency utilized in this investigation, intravaginally supplemented LAB had no impact on the prevalence of other periparturient disorders (Deng et al., 2015).

As specified by Wang and colleagues, the discovery of pediocin- producing pediococci in the vaginal microbiota of cattle may open the door to the creation of innovative preventative therapies against metritis (Wang et al., 2013).

3 Aims

The aim of this study is to examine the impact of naturally occurring inhabitants of the vaginal microbiota of cows on bovine cultured endometrial cells, *in vitro*. Additionally, to avoid uterine inflammation using beneficial bacteria from the normal microbiota of the vagina. Consequently, the reduction of the bovine subfertility, the avoidance of antibiotics that are used for prevention of uterine disorders and the incline of antimicrobial resistance.

4 Materials and Methods

4.1 Cultivation of bacteria

The applied *Trueperella pyogenes* laboratory strain was originated from cow endometritis, the *Brevibacillus agri* and *Bacillus pumilus* strains were isolated from the vaginal microbiota of healthy cow. The bacteria from the glycerol stocks were grown in brain heart infusion (BHI) broth supplemented with 10% FBS for 24 hours at 37 °C prior to the experiments. Then the bacteria were harvested by centrifugation at 3000 g for 15 minutes at room temperature.

4.2 Primary Bovine Endometrial Epithelial Cell (BEnEpC) Culture

The procedure for isolating and culturing BEnEpC has been previously reported (Betts and Hansen, 1992; Gärtner et al., 2015; Ibrahim et al., 2017). Firstly, uteri from non-pregnant cows that appeared to be in decent shape were collected from a nearby slaughterhouse. The endometrium was sliced into small pieces at several intercellular locations. The tissue mass was carefully mined before being digested for two hours at 37^{o}C in a solution containing 150 U/ml collagenase (Sigma), 150 U/ml hyaluronidase (Sigma), 200 U/ml penicillin (ThermoScientific), and 200 μ/ml streptomycin (ThermoScientific) in Hank's Balanced Salt Solution (ThermoScientific). Following centrifugation, the cell pellet was washed in epithelial cell culture medium (DMEM/Ham's F-12 medium containing 10% FBS, 55 μg/ml gentamicin, and 1.4 μg/ml amphotericin B; all from ThermoScientific). Then, the cells were seeded in 25 cm^2 culture flasks (Corning, Corning, NY, USA) and cultured for 18 hours at 37^{o}C and 5% CO_2 in a humidified incubator to promote stromal cell adhesion. After that, unattached cells were reseeded to produce a population of pure epithelial cells.

Endometrial epithelial cells cultivated during the first passage until they had more than 80% confluence. At that moment, they were placed into 24-well plates at a density of 8×10^4 cells in 500 μl medium/well for viability assays and 6-well plates at a density of 6×10^5 cells in 4 ml culture medium/well for mRNA expression assays. Confluent endometrial epithelial cell cultures were used for the experiments.

4.3 Co-culture Experiments

Co-cultures enable the investigation of cell-cell communication between several eukaryotic species or bacteria. Researchers can now more accurately simulate intricate tissue architecture because of this method (Mountcastle et al., n.d.).

In this case, primary endometrial epithelial cells were co-cultured with the bacterium in the form of live bacteria at 10^4 bacterial cells/ml.

Before the experiments the cell culture medium was removed from the confluent cell monolayer and they received two PBS washes, in order to be appropriate for co-culture experiments. Epithelial cell culture media (DMEM/Ham's F-12 medium) without antibiotics was used during the experiments. BEnEpC cultures were co-cultured with *T. pyogenes* alone or together with either *Bb. agri* or *B. pumilus*. Cells cultured with the same medium but without bacteria served as controls.

For the viability assay after 4, 6, 8, and 24 h of incubation time, the medium was aspirated, and the culture plates were washed twice with PBS. Trypan blue and PBS were added to the wells in a 1:1 mixture of 0.5% (w/v). The plates were examined with the microscope at magnification of 100×.

For the mRNA expression assay after the 4 hours incubation time the medium was removed, and the epithelial cells were given two PBS washes and then being lysed with 1 ml/well RNAzol RT reagent (Sigma). In order to form a homogenous lysate, after the reagent was added it was passed several times through a pipette tip. After the cells have been homogenized samples were stored at -80^{o}C.

4.4 RNA isolation

Firstly, 400 µl RNase-free water per sample ml was added to each sample. The samples were led to stand for 15 minutes at room temperature. Afterwards, centrifugation have been made at 12,000 g for 15 minutes at 5^{o}C. The upper supernatant (containing RNA) was transferred into a new tube. In order to allow RNA to precipitate, 400 µL of 75% ethanol (v/v) has been added. The sample was led to stand for 10 minutes in room temperature. Furthermore, the sample was centrifuged at 12,000 g for 8 minutes at 5^{o}C. The mRNA precipitate will form a white pellet on the side and the bottom of the tube which we later removed. RNA pellets were washed twice with 0.5 ml 75% ethanol (v/v) per 1 mL of supernatant used for precipitation. 50 µL of RNase-

free water was added to solubilize the RNA pellets. To finish, we measured the samples at 230, 260 and 280 nm. The ratio A260/A280 is between 1.8 and 2.1 and the ratio A260/A230 is above 1.5 for each RNA sample.

4.5 cDNA synthesis

For cDNA synthesis 1000 ng RNA template has been used. Nuclease- free water was added to RNA template up to 13µl, 1 µl random hexamer primer and 1 µl dNTP mix was added as well. Incubation at 65 °C for 5 min then 5°C for 5 min followed. Then 4 µl reaction buffer and 1 µl reverse transcriptase was also incorporated into the mixture. After centrifugation, incubation for 10 min at 25°C followed by 30 min at 50°C took place. Finally, the reaction was terminated by heating at 85 °C for 5 minutes.

4.6 Quantitative PCR

We performed the quantitative PCR analyses on the CFX Opus Real-Time PCR System (BioRad). The IL1A (F: GAAGACGAACCCGTCTTGCT, R: AAAGTCAGTGATCGAGG-GCG), IL6 (F: ACCCCAGGCAGACTACTTCT, R: CCCAGATTGGAAGCATCCGT) and CXCL8 (F: TCTCTGCAGCTCTGTGTGAAG, R: CTTGGGGTTTAGGCAGACCTC) were the tested genes, the actin beta (ACTB) (F: CACAGGCCTCTCGCCTTC, R: CCCACG-TACGAGTCCTTCTG) was used as reference gene. The final reaction volume of 20 µl contained 0.2 µM of the corresponding primers and 1× concentrated SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in nuclease-free water, and the 2 µl cDNA sample which was added directly to a PCR reaction mixture for each PCR reaction. PCR reactions were run at a thermal cycle of 95 °C for 3 minutes, followed by 40 cycles at 95 °C for 20 seconds, then at 60 °C for 30 seconds and then at 72 °C for 30 seconds. At the end of each cycle there was a 10-second-long fluorescence monitoring. After the 40 cycles melting curve analysis was performed.

4.7 Statistical analyses

Relative gene expression levels of the genes of interest were calculated by the Relative Expression Software Tool (REST) 2009 Software. Statistical analyses were performed by R version 3.6.3 (R Core Team, 2012; R: A language and environment for statistical computing). Differences between means were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc comparison using Tukey's 'Honest Significant Difference' method. Differences were considered significant if the p-value was < 0.05.

5 Results

The 4-hour-long treatment of the BEnEpC cell cultures with the *T. pyogenes, B. pumilus, Bb. agri* did not decreased the viability of the endometrial cells. Nonetheless, after longer treatment periods, 6 (Figure 1), 12, and 24 hour, the cells were seriously damaged when the supernatant of the cells contained the *T. pyogenes* bacteria. The presence of *Bb. agri* has protected the cells from the complete necrosis after 6 and 12 h of treatment.

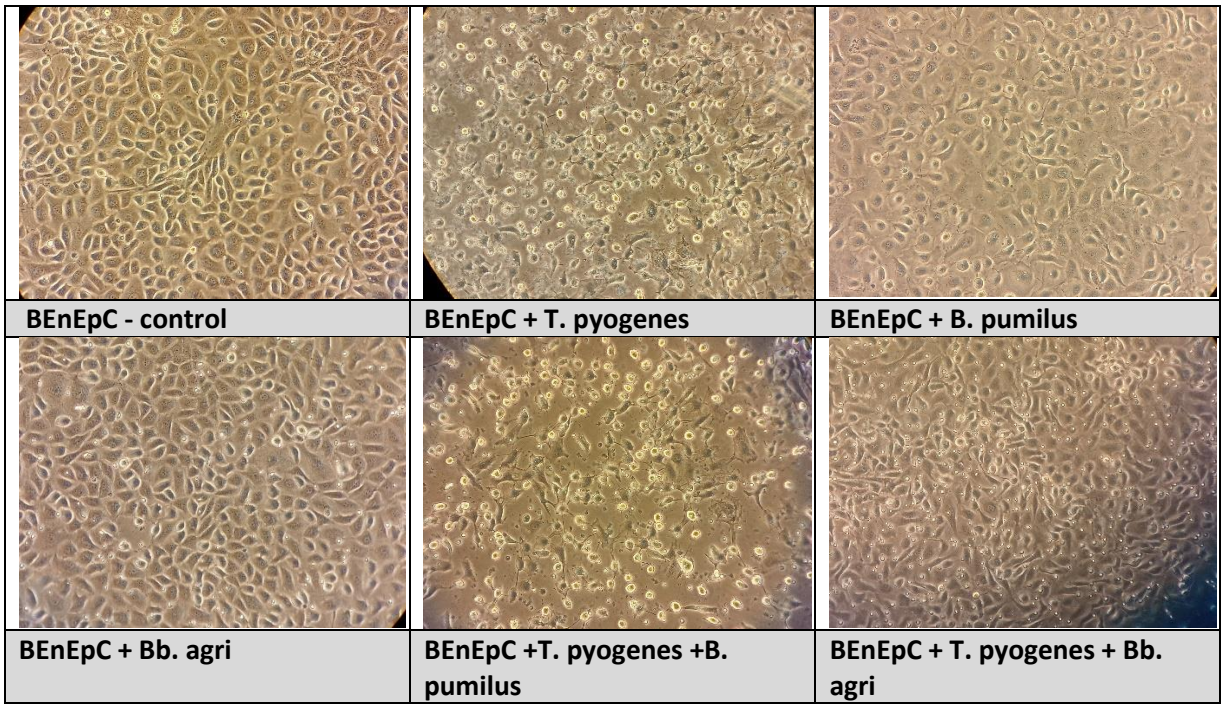

Figure 1. Representative photomicrographs of the bovine endometrial epithelial cell cultures after 6 hours of treatment, Magnification 100×

The qPCR results are presented in Figure 2. The gene expression of IL1A, IL6 and CXCL8 increased to 13-, 25-, and 18-fold, respectively, after the bovine endometrial cells were treated for 4 h with *T. pyogenes*. When *Brevibacillus agri* was added concomitantly with the *T. pyogenes* the three measured markers increased to maximum of 3,6-fold compared to the controls. After the simultaneous treatment with *T. pyogenes* and *B. pumilus* the mRNA level of IL6 increased to 18-fold, but the CXCL8 and the IL1A gene expression increased to 3.6- and 4.2-fold compared to the untreated cells.

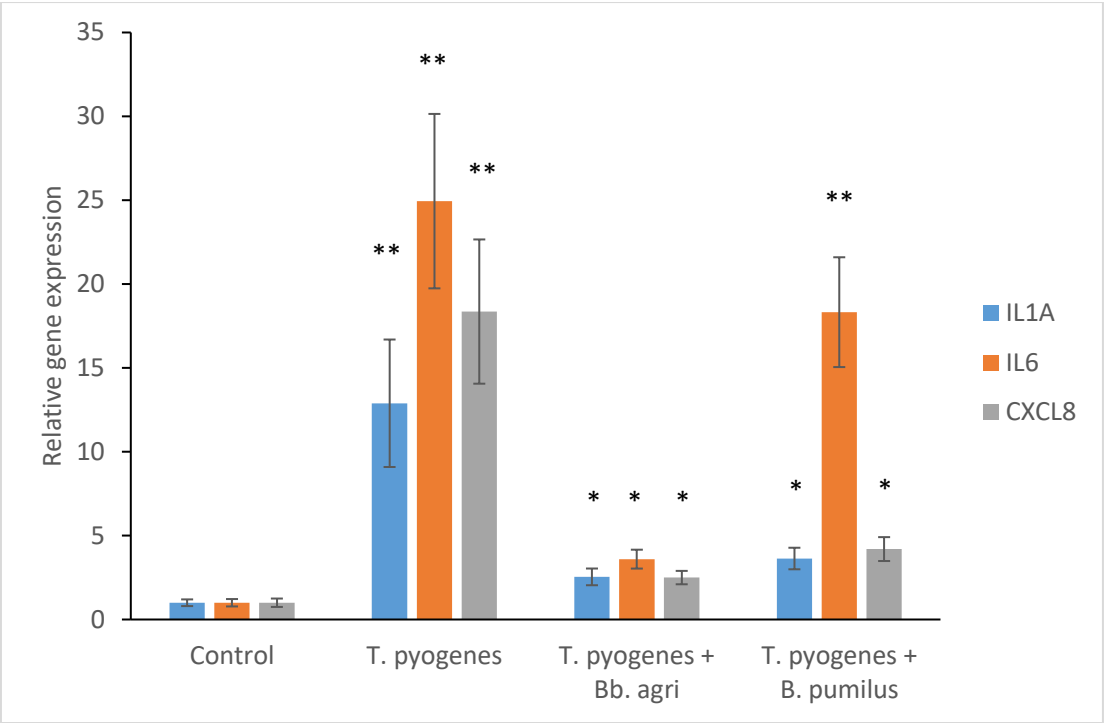

Figure 2. Relative gene expression (mRNA level) of IL1A, IL-6, and CXCL8 of BEnEpC cells exposed to 4 h treatment at 10^4 bacterial cells/ml compared to untreated controls (n=4/group). Significant differences are shown in comparison to controls (*p<0.05;**p<0.001). Data are shown as mean±SD.

Compared to the *T. pyogenes*-treated BEnEpC cells the level of IL1A, IL6, and CXCL8 was significantly lower ($p<0.001$); after the concomitant *Bb. agri* treatment. Similarly, the concurrent *B. pumilus* treatment resulted in considerably lower ($p<0.001$) IL1A and CXCL8 level, although it failed to prevent the upregulation of IL6.

6 Discussion

Bovine subfertility is among the key factors contributing to the dairy industry's enormous financial losses. Over the past few decades, numerous research has focused on the microbiota found in the uterus of postpartum cows, where the primary attention was placed on pathogenic bacteria that cause uterine disorders including metritis and endometritis. Particularly, it has been discovered that *Trueperella pyogenes* is connected to postpartum uterine inflammation (Galvão et al., 2019; Sheldon and Owens, 2017).

The current study's objective is to examine the impact of naturally occurring occupants of cow's vaginal microbiota on cultured bovine endometrial cells, in vitro. Moreover, to prevent uterine inflammation using advantageous bacteria from the vagina's normal microbiota. Therefore, primary bovine endometrial cells were co-cultured with *Trueperella pyogenes* and then as therapy, *Trueperella pyogenes* was co-cultured with *Brevibacillus agri* and *Bacillus pumilus,* bacteria isolated from healthy cow's uterus. This study followed the hypothesis that those bacteria can reduce the killing effect of *Trueperella pyogenes* therefore can be considered beneficial for the prevention of diseases in postpartum cows.

The results indicated that *Trueperella pyogenes* treatment greatly increased the gene expression of IL1A, IL6 and CXCL8. These gene expression levels appear to be too high, it should be considered for future studies, that shorter treatment period should be applied. Also because the 6-hour treatment proved to be too long in the viability studies. Nonetheless, *Trueperella pyogenes* with *Brevibacillus agri* prevented the increase of all three markers, while *Trueperella pyogenes* with *Bacillus pumilus* prevented the increase of CXCL8 and IL1A, but not the IL6.

The development of uterine bacterial infections is influenced by both the local uterine immune response and the pathogenic potential of the invasive bacteria. Primary endometrial epithelial cells, have the ability to trigger an immune response by producing various cytokines (Ibrahim et al., 2017). The endometrium's immune response begins with the identification of invasive infections, then it produces pro-inflammatory cytokines such as interleukins and chemokines to draw polymorphonuclear neutrophils (PMNs) to the lumen of the uterus for bacterial elimination (Gärtner et al., 2015).

The protein (IL-1a) encoded by IL1A, is a member of the interleukin 1 cytokine family and it is principally engaged in pro-inflammatory immunological responses and hematopoiesis. It has

been discovered that endometriosis has been linked to elevated levels of inflammatory markers in the blood. Some researchers have observed a connection between endometriosis and autoimmune illnesses (Sapkota et al., 2015). In contrast to healthy cows, cows with clinical and subclinical endometritis showed higher mRNA expression of IL1A in endometrial cytobrush samples (Gärtner et al., 2015). Likewise, *Trueperella pyogenes* may cause the uterus to experience pro-inflammatory symptoms, including neutrophil transmigration and mucopurulent discharge (Amos et al., 2014). The pleiotropic cytokine IL-6 is both pro- and anti-inflammatory. It is produced by T and B lymphocytes, fibroblasts, monocytes, endothelial and malignant cells as a reaction to various pro-inflammatory stimuli (Milovanović et al., 2019). Conversely, IL-6 excessive production can lead to autoimmune and inflammatory conditions (Uciechowski and Dempke, 2020). It has been determined that the chemokine CXCL8 may regulated endometrial stromal cells, although it is unknown how CXCL8 controls endometrial stromal cells survival through the pathogenesis of endometriosis (Li et al., 2012). According to a research, blood leukocytes from cows with subclinical endometritis expressed more copies of CXCL8 than the ones with non-subclinical endometritis (Düvel et al., 2014). Cows with subclinical endometritis had higher relative mRNA expression for CXCL8 than healthy cows did. Unexpectedly no change has been seen for IL-6 mRNA expression between healthy cows and cows with subclinical endometritis (Bonsale et al., 2018). However, IL-6 and CXCL8, which are required to start an efficient inflammatory reaction against infection, have been linked to numerous organ system failure and death when produced in excess (Bonsale et al., 2018). These findings can explain the result that *Trueperella pyogenes* treatment greatly increased the gene expression of IL1A, IL6 and CXCL8.

Several studies have suggested that intravaginal *Lactobacillus spp.* have reduced uterine infections, enhanced immune responses (Deng et al., 2015; Otero et al., 2006; Peter et al., 2018; Rosales and Ametaj, 2021). Numerous immunological benefits of LAB have been demonstrated to be connected with lactic acid. Thus, it has been proven that lactic acid inhibits the host's production of the inflammatory mediators IL-6 and IL-8 in response to exposure to pathogenic bacteria (Rosales and Ametaj, 2021). Nonetheless, a research found that *Lactobacillus ruminis* and *Lactobacillus amylovorus,* increased IL-1A and IL-6 expression in co-cultured endometrial epithelial cells more than the control cells. These outcomes demonstrate Lactobacilli's capacity to promote endometrial immune response (Gärtner et al., 2015).

These findings can partly clarify our findings that *T. pyogenes* with *Brevibacillus agri* prevented the increase of all three markers and *T. pyogenes* with *Bacillus pumilus* prevented the increase of CXCL8 and IL1A.

A study research which investigated the first inflammatory response of the endometrium *in vivo* and *in vitro*, discovered that IL-1A and IL-6 had considerably greater levels or mRNA expression when *Bacillus pumilus* was present (Gärtner et al., 2016). These findings were controversial to our findings as *Bacillus pumilus* prevented the increase of CXCL8 and IL1A, but not IL6.

In vitro models have been used to study the impact of uterine infections on endometrial epithelial cells in great detail (Davies et al., 2008; Gärtner et al., 2016). This could not accurately reflect the host pathogen interactions that occur in vivo, where endometrial epithelial cells and immune cells also interact (Borges et al., 2012). Our findings support that naturally occurring occupants of cow's vaginal microbiota can inhibit the killing effect of *T. pyogenes*, which was found to be associated with postpartum uterine infections. Further *in vivo* research is suggested to prove our findings in live animals.

Antibiotics, hormones, and antiseptic agents are increasingly used to treat cows with postpartum uterine inflammation. Particular issues occur owing to the increased prevalence of microbial resistance, drug residues in milk and meat and rising costs of production, all of which point to ineffectiveness of such treatments. Intravaginal probiotic microorganisms have been shown from previous literature to decrease uterine infections, improve immunological responses, and improve milk production. In this present study we tested *Trueperella pyogenes* with *Brevibacillus agri* and with *Bacillus pumilus*. Both bacteria (*Brevibacillus agri* and *Bacillus pumilus*) appear to prevent the inflammatory processes induced by *T. pyogenes*. In case of *Brevibacillus agr*i the results are more encouraging since it prevented all of the 3 markers (IL-1A, IL-6 and CXCL8) when *Bacillus pumilus* appeared to prevent only 2 of them (IL1A and CXCL8). Our results appear to be promising but more research is suggested in order to prove these results *in vivo*.

7 Abstract

One of the main causes of the dairy industry's significant financial losses is bovine subfertility. Even though bacterial infections frequently contribute to reproductive problems, limited data is describing the normal vaginal microbiota of the cow. Determining the microbial colonies found in the reproductive canal of the cow may allow us to better understand the physiology of the animal's reproductive system, which has great commercial significance. The postpartum uterine inflamed cows are now treated with antibiotics, hormones, and antiseptic agents. Specific concerns arise since there is high incidence of microbial resistance, drug residues in milk and meat production cost increase which indicates lack of efficiency of those treatments. As suggested from previous studies, intravaginal probiotic bacteria have reduced uterine infections, enhanced immune responses, and increased milk production. The aim of the present study is to investigate the effects of naturally occurring inhabitants of the vaginal microbiota of cows on cultured bovine endometrial cells, *in vitro*. Furthermore, to prevent the inflammation of the uterus with beneficial bacteria from normal microbiota of the vagina. Primary bovine endometrial cells were co-cultured with *Trueperella pyogenes,* which according to previous literature was found to be linked to postpartum uterine illness. For prevention of *Trueperella pyogenes*-caused cell-damage; potentially beneficial bacteria, which was isolated from healthy cow's reproductive tract, were added to the cell cultures. After the treatment mRNA was isolated from the endometrial epithelial cells. Following reverse transcription PCR, the level of selected inflammatory markers was assessed via quantitative PCR. Our results suggest that the naturally occurring inhabitants of the vaginal microbiota of cows can be beneficial in eliminating the harmful effects caused by invading pathogens. Further *in vivo* research is needed in order to prove our *in vitro* findings.

8 Összefoglaló

A tejtermelő ágazat jelentős pénzügyi veszteségeinek egyik fő oka a szarvasmarhák csökkent fertilitása. Annak ellenére, hogy a bakteriális fertőzések gyakran hozzájárulnak a szaporodásbiológiai problémákhoz, korlátozott számú forrás írja le, hogy mit tekinthetünk tehénben normál hüvelyi mikrobiotának. A tehenek ivarszervében található mikrobiális közösségek meghatározása lehetővé teszi, hogy jobban megértsük az állat reproduktív rendszerének élettani vonatkozásait, amelynek nagy gazdasági jelentősége lehet. Jelenleg, az ellést követő méhgyulladást antibiotikumokkal, hormonokkal és antiszeptikumokkal kezelik. Aggodalomra ad okot az antimikrobiális rezisztencia növekvő elterjedése, a tejben és a húsban kimutatható gyógyszermaradványok gyakori előfordulása, valamint a termelési költségek növekedése, mindez a kezelések nem megfelelő hatékonyságát jelzi. Korábbi vizsgálatok kimutatták, hogy az intravaginálisan alkalmazott probiotikus baktériumok csökkentik a méhfertőzés kockázatát, fokozzák az immunválaszt, valamint növelik a tejtermelés hatékonyságát. Jelen tanulmány célja a tehenek hüvelyében természetesen előforduló mikrobák hatásának vizsgálata szarvasmarha endometriális sejttenyészeteken *in vitro*. Amely jótékony baktériumok a továbbiakban élő állatokban alkalmazva alkalmasak lehetnek a méhgyulladás kialakulásának megelőzésében. Primer szarvasmarha endometrium sejteket *Trueperella pyogenes* baktérium kultúrával együtt tenyésztettünk, ez a patogén mikroba az ellés utáni méh megbetegedéseihez kapcsolódik. A *Trueperella pyogenes* által okozott sejtkárosodás megelőzésére olyan potenciálisan jótékony baktériumokat adtunk a sejttenyészetekhez, amelyeket egészséges tehén reproduktív rendszeréből izoláltunk. A kezelést követően mRNS-t izoláltuk az endometriális sejttenyészetekből. A reverz transzkripciót követően a kiválasztott gyulladásos markerek szintjét kvantitatív polimeráz-láncreakció segítségével határoztuk meg. Eredményeink azt mutatják, hogy a tehén hüvelyi mikrobiota természetesen előforduló tagjai hasznosak lehetnek a méhbe bekerülő kórokozók által okozott káros hatások kiküszöbölésében. További *in vivo* vizsgálatokra van szükség az *in vitro* mérésekből származó megállapításaink igazolásához.

9 Bibliography

Aidara-Kane, A., 2012. Containment of antimicrobial resistance due to use of antimicrobial agents in animals intended for food: WHO perspective. Rev. Sci. Tech. Int. Off. Epizoot. 31, 277–287. https://doi.org/10.20506/rst.31.1.2115

Amos, M.R., Healey, G.D., Goldstone, R.J., Mahan, S.M., Düvel, A., Schuberth, H.-J., Sandra, O., Zieger, P., Dieuzy-Labaye, I., Smith, D.G.E., Sheldon, I.M., 2014. Differential Endometrial Cell Sensitivity to a Cholesterol-Dependent Cytolysin Links Trueperella pyogenes to Uterine Disease in Cattle1. Biol. Reprod. 90, 54, 1–13. https://doi.org/10.1095/biolreprod.113.115972

Appiah, M.O., Wang, J., Lu, W., 2020. Microflora in the Reproductive Tract of Cattle: A Review. Agriculture 10, 232. https://doi.org/10.3390/agriculture10060232

Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gómez-Llorente, C., Gil, A., 2012. Probiotic Mechanisms of Action. Ann. Nutr. Metab. 61, 160–174. https://doi.org/10.1159/000342079

Betts, J.G., Hansen, P.J., 1992. Regulation of prostaglandin secretion from epithelial and stromal cells of the bovine endometrium by interleukin-1 beta, interleukin-2, granulocyte-macrophage colony stimulating factor and tumor necrosis factor-alpha. Life Sci. 51, 1171–1176. https://doi.org/10.1016/0024-3205(92)90520-y

Bonsale, R., Seyed Sharifi, R., Dirandeh, E., Hedayat, N., Mojtahedin, A., Ghorbanalinia, M., Abolghasemi, A., 2018. Endocannabinoids as endometrial inflammatory markers in lactating Holstein cows. Reprod. Domest. Anim. 53, 769–775. https://doi.org/10.1111/rda.13169

Borges, Á.M., Healey, G.D., Sheldon, I.M., 2012. Explants of Intact Endometrium to Model Bovine Innate Immunity and Inflammation Ex Vivo. Am. J. Reprod. Immunol. 67, 526–539. https://doi.org/10.1111/j.1600-0897.2012.01106.x

Davies, D., Meade, K.G., Herath, S., Eckersall, P.D., Gonzalez, D., White, J.O., Conlan, R.S., O'Farrelly, C., Sheldon, I.M., 2008. Toll-like receptor and antimicrobial peptide expression in the bovine endometrium. Reprod. Biol. Endocrinol. 6, 53. https://doi.org/10.1186/1477-7827-6-53

Deng, Q., Odhiambo, J.F., Farooq, U., Lam, T., Dunn, S.M., Ametaj, B.N., 2015. Intravaginal Lactic Acid Bacteria Modulated Local and Systemic Immune Responses and Lowered the Incidence of Uterine Infections in Periparturient Dairy Cows. PLOS ONE 10, e0124167. https://doi.org/10.1371/journal.pone.0124167

Düvel, A., Maaß, J., Heppelmann, M., Hussen, J., Koy, M., Piechotta, M., Sandra, O., Smith, D.G.E., Sheldon, I.M., Dieuzy-Labaye, I., Zieger, P., Schuberth, H.J., 2014. Peripheral blood leukocytes of cows with subclinical endometritis show an altered cellular composition and gene expression. Theriogenology 81, 906–917. https://doi.org/10.1016/j.theriogenology.2014.01.007

Elad, D., Beckwith-Cohen, B., n.d. Variations in Vaginal pH in Dairy Cattle Associated with Parity and the Periparturient Period. Isr. J. Vet. Med.

Galvão, K.N., Bicalho, R.C., Jeon, S.J., 2019. Symposium review: The uterine microbiome associated with the development of uterine disease in dairy cows. J. Dairy Sci. 102, 11786–11797. https://doi.org/10.3168/jds.2019-17106

Gärtner, M.A., Bondzio, A., Braun, N., Jung, M., Einspanier, R., Gabler, C., 2015. Detection and characterisation of Lactobacillus spp. in the bovine uterus and their influence on bovine endometrial epithelial cells in vitro. PloS One 10, e0119793. https://doi.org/10.1371/journal.pone.0119793

Gärtner, M.A., Peter, S., Jung, M., Drillich, M., Einspanier, R., Gabler, C., 2016. Increased mRNA expression of selected pro-inflammatory factors in inflamed bovine endometrium in vivo as well as in endometrial epithelial cells exposed to Bacillus pumilus in vitro. Reprod. Fertil. Dev. 28, 982–994. https://doi.org/10.1071/RD14219

Haimerl, P., Arlt, S., Borchardt, S., Heuwieser, W., 2017. Antibiotic treatment of metritis in dairy cows—A meta-analysis. J. Dairy Sci. 100, 3783–3795. https://doi.org/10.3168/jds.2016-11834

Haimerl, P., Heuwieser, W., 2014. Invited review: Antibiotic treatment of metritis in dairy cows: A systematic approach. J. Dairy Sci. 97, 6649–6661. https://doi.org/10.3168/jds.2014-8462

Hristov, A.N., Callaway, T.R., Lee, C., Dowd, S.E., 2012. Rumen bacterial, archaeal, and fungal diversity of dairy cows in response to ingestion of lauric or myristic acid. J. Anim. Sci. 90, 4449–4457. https://doi.org/10.2527/jas.2011-4624

Ibrahim, M., Peter, S., Wagener, K., Drillich, M., Ehling-Schulz, M., Einspanier, R., Gabler, C., 2017. Bovine Endometrial Epithelial Cells Scale Their Pro-inflammatory Response In vitro to Pathogenic Trueperella pyogenes Isolated from the Bovine Uterus in a Strain-Specific Manner. Front. Cell. Infect. Microbiol. 7, 264. https://doi.org/10.3389/fcimb.2017.00264

Inas, T.U., Suprihati, E., Madyawati, S.P., Sardjito, T., Utomo, B., Srianto, P., 2017. Influence of pH Vaginal Mucosa When Artifical Insemination of Non Return Rate (NRR) and Conception Rate (CR) on Dairy Cow in KSU Tunas Setia Baru Pasuruan East Java. Int. J. Dev. Res. 7, 15540–15542.

Jeon, S.J., Lima, F.S., Vieira-Neto, A., Machado, V.S., Lima, S.F., Bicalho, R.C., Santos, J.E.P., Galvão, K.N., 2018. Shift of uterine microbiota associated with antibiotic treatment and cure of metritis in dairy cows. Vet. Microbiol. 214, 132–139. https://doi.org/10.1016/j.vetmic.2017.12.022

Laguardia-Nascimento, M., Branco, K.M.G.R., Gasparini, M.R., Giannattasio-Ferraz, S., Leite, L.R., Araujo, F.M.G., Salim, A.C. de M., Nicoli, J.R., de Oliveira, G.C., Barbosa-Stancioli, E.F., 2015. Vaginal Microbiome Characterization of Nellore Cattle Using Metagenomic Analysis. PLOS ONE 10, e0143294. https://doi.org/10.1371/journal.pone.0143294

Li, M.-Q., Luo, X.-Z., Meng, Y.-H., Mei, J., Zhu, X.-Y., Jin, L.-P., Li, D.-J., 2012. CXCL8 enhances proliferation and growth and reduces apoptosis in endometrial stromal cells in an autocrine manner via a CXCR1-triggered PTEN/AKT signal pathway. Hum. Reprod. Oxf. Engl. 27, 2107–2116. https://doi.org/10.1093/humrep/des132

Lima, F.S. de, 2020. Recent advances and future directions for uterine diseases diagnosis, pathogenesis, and management in dairy cows. Anim. Reprod. 17. https://doi.org/10.1590/1984-3143-AR2020-0063

Ma, Z., Ginn, A., Kang, M., Galvão, K.N., Jeong, K.C., 2018. Genomic and Virulence Characterization of Intrauterine Pathogenic Escherichia coli With Multi-Drug Resistance Isolated From Cow Uteri With Metritis. Front. Microbiol. 9.

Manishimwe, R., Nishimwe, K., Ojok, L., 2017. Assessment of antibiotic use in farm animals in Rwanda. Trop. Anim. Health Prod. 49, 1101–1106. https://doi.org/10.1007/s11250-017-1290-z

McEwen, S.A., Angulo, F.J., Collignon, P.J., Conly, J., 2017. Potential unintended consequences associated with restrictions on antimicrobial use in food-producing animals, WHO Guidelines on Use of Medically Important Antimicrobials in Food-Producing Animals. World Health Organization.

McEwen, S.A., Angulo, F.J., Collignon, P.J., Conly, J.M., 2018. Unintended consequences associated with national-level restrictions on antimicrobial use in food-producing animals. Lancet Planet. Health 2, e279–e282. https://doi.org/10.1016/S2542-5196(18)30138-4

McEwen, S.A., Collignon, P.J., 2018. Antimicrobial Resistance: a One Health Perspective. Microbiol. Spectr. 6, 6.2.10. https://doi.org/10.1128/microbiolspec.ARBA-0009-2017

Milovanović, J., Todorović-Raković, N., Radulovic, M., 2019. Interleukin-6 and interleukin-8 serum levels in prognosis of hormone-dependent breast cancer. Cytokine 118, 93–98. https://doi.org/10.1016/j.cyto.2018.02.019

Mountcastle, S.E., Cox, S.C., Sammons, R.L., Jabbari, S., Shelton, R.M., Kuehne, S.A., n.d. A review of co-culture models to study the oral microenvironment and disease. J. Oral Microbiol. 12, 1773122. https://doi.org/10.1080/20002297.2020.1773122

NADIS Animal Health Skills - Fertility in dairy herds [WWW Document], n.d. URL https://www.nadis.org.uk/disease-a-z/cattle/fertility-in-dairy-herds/ (accessed 10.7.22).

Oliveira, E.B. de, Cunha, F., Daetz, R., Figueiredo, C.C., Chebel, R.C., Santos, J.E., Risco, C.A., Jeong, K.C., Machado, V.S., Galvão, K.N., 2020. Using chitosan microparticles to treat metritis in lactating dairy cows. J. Dairy Sci. 103, 7377–7391. https://doi.org/10.3168/jds.2019-18028

Olson, J.D., Ball, L., Mortimer, R.G., 1984. Therapy of Postpartum Uterine Infections. Am. Assoc. Bov. Pract. Conf. Proc. 85–88. https://doi.org/10.21423/aabppro19847282

Ong, C.T., Turni, C., Blackall, P.J., Boe-Hansen, G., Hayes, B.J., Tabor, A.E., 2021. Interrogating the bovine reproductive tract metagenomes using culture-independent approaches: a systematic review. Anim. Microbiome 3, 41. https://doi.org/10.1186/s42523-021-00106-3

Otero, C., Saavedra, L., Silva de Ruiz, C., Wilde, O., Holgado, A. r., Nader-Macías, M. e., 2000. Vaginal bacterial microflora modifications during the growth of healthy cows. Lett. Appl. Microbiol. 31, 251–254. https://doi.org/10.1046/j.1365-2672.2000.00809.x

Otero, M. c., Morelli, L., Nader-Macías, M. e., 2006. Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle. Lett. Appl. Microbiol. 43, 91–97. https://doi.org/10.1111/j.1472-765X.2006.01914.x

Peter, S., Gärtner, M.A., Michel, G., Ibrahim, M., Klopfleisch, R., Lübke-Becker, A., Jung, M., Einspanier, R., Gabler, C., 2018. Influence of intrauterine administration of Lactobacillus buchneri on reproductive performance and pro-inflammatory endometrial mRNA expression of cows with subclinical endometritis. Sci. Rep. 8, 5473. https://doi.org/10.1038/s41598-018-22856-y

Pulfer, K.W., Riese, R.L., n.d. Treatment of Postpartum Metritis in Dairy Cows. Iowa State Univ. Vet. 6.

Raheel, I.A.E.R., Hassan, W.H., Salem, S.S.R., Salam, H.S.H., 2020. Biofilm forming potentiality of Escherichia coli isolated from bovine endometritis and their antibiotic resistance profiles. J. Adv. Vet. Anim. Res. 7, 442–451. https://doi.org/10.5455/javar.2020.g440

Roper, D., 2014. Characterization of the Reproductive Tract in Recipients for Bovine Embryo Transfer: pH and Bacterial Presence. Dr. Diss.

Rosales, E.B., Ametaj, B.N., 2021. Reproductive Tract Infections in Dairy Cows: Can Probiotics Curb Down the Incidence Rate? Dairy 2, 40–64. https://doi.org/10.3390/dairy2010004

Sapkota, Y., Low, S.-K., Attia, J., Gordon, S.D., Henders, A.K., Holliday, E.G., MacGregor, S., Martin, N.G., McEvoy, M., Morris, A.P., Takahashi, A., Scott, R.J., Kubo, M., Zondervan, K.T., Montgomery, G.W., Nyholt, D.R., 2015. Association between endometriosis and the interleukin 1A (IL1A) locus. Hum. Reprod. Oxf. Engl. 30, 239–248. https://doi.org/10.1093/humrep/deu267

Sheldon, I.M., Lewis, G.S., LeBlanc, S., Gilbert, R.O., 2006. Defining postpartum uterine disease in cattle. Theriogenology 65, 1516–1530. https://doi.org/10.1016/j.theriogenology.2005.08.021

Sheldon, I.M., Owens, S.E., 2017. Postpartum uterine infection and endometritis in dairy cattle. Anim. Reprod. 14, 622–629. https://doi.org/10.21451/1984-3143-AR1006

Sheldon, I.M., Price, S.B., Cronin, J., Gilbert, R.O., Gadsby, J.E., 2009. Mechanisms of infertility associated with clinical and subclinical endometritis in high producing dairy cattle. Reprod. Domest. Anim. Zuchthyg. 44 Suppl 3, 1–9. https://doi.org/10.1111/j.1439-0531.2009.01465.x

Sheldon, I.M., Rycroft, A.N., Dogan, B., Craven, M., Bromfield, J.J., Chandler, A., Roberts, M.H., Price, S.B., Gilbert, R.O., Simpson, K.W., 2010. Specific Strains of Escherichia coli Are Pathogenic for the Endometrium of Cattle and Cause Pelvic Inflammatory Disease in Cattle and Mice. PLoS ONE 5, e9192. https://doi.org/10.1371/journal.pone.0009192

Sheldon, I.M., Williams, E.J., Miller, A.N.A., Nash, D.M., Herath, S., 2008. Uterine diseases in cattle after parturition. Vet. J., Special Issue: Production Diseases of the Transition Cow 176, 115–121. https://doi.org/10.1016/j.tvjl.2007.12.031

Smith, B.I., Arthur Donovan, G., Risco, C., Littell, R., Young, C., Stanker, L.H., Elliott, J., 1998. Comparison of Various Antibiotic Treatments for Cows Diagnosed with Toxic Puerperal Metritis. J. Dairy Sci. 81, 1555–1562. https://doi.org/10.3168/jds.S0022-0302(98)75721-2

Swartz, J.D., Lachman, M., Westveer, K., O'Neill, T., Geary, T., Kott, R.W., Berardinelli, J.G., Hatfield, P.G., Thomson, J.M., Roberts, A., Yeoman, C.J., 2014. Characterization of the Vaginal Microbiota of Ewes and Cows Reveals a Unique Microbiota with Low Levels of Lactobacilli and Near-Neutral pH. Front. Vet. Sci. 1.

Uciechowski, P., Dempke, W.C.M., 2020. Interleukin-6: A Masterplayer in the Cytokine Network. Oncology 98, 131–137. https://doi.org/10.1159/000505099

Wang, Y., Ametaj, B.N., Ambrose, D.J., Gänzle, M.G., 2013. Characterisation of the bacterial microbiota of the vagina of dairy cows and isolation of pediocin-producing Pediococcus acidilactici. BMC Microbiol. 13, 19. https://doi.org/10.1186/1471-2180-13-19

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I.Acknowledgements and any other declarations

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Appendix 5. Declaration regarding TDK research paper-thesis equivalence

DECLARATION

I hereby declare that the thesis entitled ‘‘Effects of the naturally occurring inhabitants of the vaginal microbiota of cows on cultured endometrial cells’’

is identical in terms of content and formal requirements to the TDK research paper submitted in 2022.

Date:

04/11/2023

……Vania Yiannaki……

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