University of Veterinary Medicine Budapest
Department of Pharmacology and Toxicology

Identification of naturally occurring inhabitants of vaginal microbiota in cows and determination of their antibiotic sensitivity

By Jad El Hawly

Supervisors:
Dr. György Csikó
associate professor

Dr. Orsolya Palócz
research fellow

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List of Abbreviations

BHI	Brain heart infusion
BV	Bacterial vaginosis
CM	Cervical mucus
DNA	Deoxyribonucleic acid
FDA	United States Food and Drug Administration
FSH	Follicle-stimulating hormone
HGT	Horizontal gene transfer
HP	Haptoglobin
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MIC	Minimum inhibitory concentration
NSAIDs	Non-steroidal anti-inflammatory medicines
PCR	Polymerase chain reaction
PMF	Peptide mass fingerprint
RNA	Ribonucleic acid
RFM	Retained fetal membranes
Spp.	Species
VMB	Vaginal microbiota
WHO	World Health Organization

1. Introduction

1.1. Background

Previous advancements in the sequence technology of DNA enable thorough characterizations of the uterus microbiome of dairy cows with the aim of determining a fundamental microbial community and connections of certain taxa with the presence or absence of reproductive illness. The vaginal microbiome of postnatal cattle has been the subject of recent metagenomics investigations that have described the variety of bacterial communities. These researches have revealed striking changes in structure across normal cows and cows with fertility disorders [1]. Additionally, it was discovered that illness with harmful strains of *Escherichia coli* upset the endometrial microbiota's normal equilibrium, making it easier for *Trueperella* and *Fusobacteria* species to contaminate the patient later. This postpartum bacterial dysregulation is of special importance since uterine illness is a significant source of financial loss in high-yield dairy cattle [2].

The extensive usage of antimicrobial products has been blamed for the rise in the prevalence of bacterial resistant to antibiotics [3]. In reality, surroundings where drugs are utilized have a high prevalence of antibiotic-resistant gut bacteria. In environments where contact to antibiotics is predicted to be uncommon or absent, resistant microorganisms have also been discovered. Antibiotic-resistant bacteria have been found in the digestive tracts of animals in the wild [4, 5]. Since the usage of antibiotics not only favors the survival of resistant organisms but also, in certain circumstances, can encourage the transmission of mobility components, the claim that antibiotic use increases the prevalence of antibiotic-resistant microbes appears logical. Tetracycline, for instance, promotes the transmission of conjugative transposons, these elements account for the majority of antibiotic resistance transfer [6].

Seven decades following the first discovery of antibiotics, resistance to these life-saving medications has grown to be a serious worldwide health concern. Antibiotic-resistant illnesses caused approximately 25,000 fatalities in Europe in 2007; in the United States, this number was close to 100,000, with an anticipated price of United States $21-34 billion. [7]. Most areas of healthcare are affected by antibiotic-resistant organisms, and even the smallest infection may be difficult to treat if the germs are resistant to antibiotics [6].

Microbiota in and on bodies of different species can have a significant impact on the biology of the host. Most studies have focused on the microbiota of gut, skin, and mouth, very little is known about normal vaginal microbiota in livestock, even though many common reproductive disorders are associated with bacterial infections. The characteristic bovine uterine diseases such as metritis and endometritis can be the results of colonization through the extrinsic and ascending pathways to the vagina, furthermore, bacteria can also penetrate from the gut to the uterus. Maintenance of the healthy vaginal microbiota e.g., by using probiotic products may prevent postpartum infections in cows.

This raises the concern on the use of the antibiotics for cows and cattle illnesses and its effect on the vaginal and uterine microbiota of these cows.

2. Literature Review

2.1. Dairy cows

A colony of benign, beneficial, and pathogenic bacteria that populate the vagina is referred to as the vaginal microbiota (VMB). In a healthy female reproductive tract of humans, *lactobacilli* are among the predominant organisms that play a crucial role in preventing many vaginal and reproductive systemic diseases. Any fluctuations or damage to such microbiota could cause uterine and vaginal infections.

The control of dairy farms health is going through a time of profound transformation globally. The abolition of quotas (inside Europe) and the considerable expansion of cow and farm area are only a few of the numerous factors driving this transformation. Many European nations are increasing their dairy productivity and yield of the elimination of restrictions. For instance, Ireland has ambitions to raise dairy production by 50%, which will be. accomplished by increasing the size of the herd and the amount of milk produced by each cow [8].

2.2. Problems facing cow breeding

Generally, in comparison to other farm animals that give birth to the offspring or lay eggs, cow breeding (which can give birth to one calf per year only) is less effective [9-12]. For commercially viable beef and dairy output, as well as cow substitutes, the number of calves produced and reared every breeding cycle is unavoidably crucial. Thus, it is crucial for the cattle businesses to keep bovine breeding efficiency at its peak. Performance in bovine fertility is a complex feature that is influenced by both viral and non-infectious elements. Some of the non-infectious variables include diet, environmental elements, and genomic variance in fertility [13, 14]. Inflammatory response and damaged breeding abilities can result from contagious elements, which are mainly connected to continual microbial populations and can take many different forms, such as warped and twisted reproductive cycles, decreased conception rates, higher likelihood of abortion, fetal death, and prolonged calving seasonal changes.

In addition to microbial infection after calving, infections from pollution that comes that enter the cow's reproductive system throughout coupling and artificial insemination [14-16]. The

effectiveness of cow reproduction was decreased by the development of opportunistic infections in the cattle reproductive system. The control of hormonal levels is disrupted by microbial pathogens in the reproductive system of bovine. For instance, estradiol levels were decreased and caused the onset of ovulation to delay in two different investigations utilizing either cows with endometritis or those exposed to bacterial lipopolysaccharide (LPS) [17].

Additionally, it has been demonstrated that bacterial LPS exposure alters the uterus microenvironment by reducing progesterone and interfering with the control of luteinizing hormone and prostaglandin F2 [18–20]. The infiltration of cow reproductive system by pathogenic microorganisms and their toxins causes inflammation, inducing host immune reactions and destroying the endometrium muscle, creating unfavorable circumstances for the transfer of sperm cells and development of the embryo [21]. Opportunistic pathogen amplification is also detrimental to early embryogenesis because it raises the likelihood of early embryo mortality, miscarriage, or the delivery of a calf that is malformed or has a chronic infection. Using conventional culture methods, species of bacteria such as *Escherichia coli, Fusobacterium spp., Prevotella spp*., and *Trueperella pyogenes* were recovered from the endometrium of cattle. According to certain theories, these bacteria are the pathogen causing postpartum endometrial pathology [22,23].

2.3. Bacterial communities and pH of cow vagina and uterus

In general, the normal vaginal microbiome, *lactobacilli* frequently take the lead. The synthesis of lactate by the *lactobacilli* in this network is thought to be crucial for maintaining vaginal stability because it keeps the pH of the vagina down (pH 4.5), which significantly prevents many vaginal infections [24]. Given the possible role of vaginal *lactobacilli* in reducing the likelihood of difficulties associated to pregnancy, it is intriguing to observe that not every primate and all humans have the same trend of *Lactobacillus* being the most common bacteria in the vagina. Similar to this, the few culture-based investigations on cattle have shown lesser populations of *Lactobacillus spp.* in the vagina of both cows and ewes than other microbial species [25, 26]. More frequently found in the cow vagina are *Enterococcus spp.*, *Staphylococcus spp.*, and *Streptococcus spp.*, whereas the ewe vagina has *Bacillus spp.*, *Corynebacterium spp.*, *Escherichia spp.*, *Staphylococcus spp.*, and *Streptococcus spp*.

There are a variety of aerobic, facultatively anaerobic, and obligately anaerobic microbes in a normal cow reproductive system. The normal microbiota of the vagina of cows was reported to have small amounts of *lactobacilli* and to be dominated by *Enterobacteriaceae*. Total microbial counts in genital mucus were poor, and the makeup of the cattle vaginal microbiota on a species level was very changeable [27, 28]. Therefore, bacteria discovered in the microbiota are more likely to be environmental pollutants *(Bacillus spp.)*, wither coming from cow skin *(Staphylococcus spp.)*, or in the feces *(Escherichia coli,* lactic acid bacteria*)*, rather than being a steady biota native to the reproductive system.

2.4. Peri- and postpuerperal disease of cows

Dairy cattle may be more vulnerable to increase in bacterial populations and metritis after delivery if there isn't a competing commensal vaginal microbiome [29, 30]. In fact, when comparing samples from uninfected cows to those from infected ones, quantitative PCR showed a significant increase in bacterial count in infected cows, especially of *Enterobacteriaceae* and *Escherichia coli*.

The alterations in bacterial abundance and the hormonal changes occurring in the cow reproductive system at various periods of the breeding cycle may be related, as shown by metagenomic-based studies of the cattle reproductive tract. In a study describing the vaginal microbiomes of heifers, non-pregnant cows, primiparous, and multiparous cows, it was shown that pregnant cows had a reduced bacterial makeup and a greater archaeal frequency [31,32]. Since progesterone predominates throughout pregnancy, there may be a relationship between the bacterial community's composition and progesterone levels. However, throughout the oestrus synchronization procedure, alterations in microbial populations in the uterus were also noted, supporting the impact of hormonal changes on the bacterial composition.

The conventional wisdom holds that the environment of the endometrium is sterile, especially while a female is pregnant. It is now understood that bacteria in the childbirth region might enter the cow's uterus during birthing [33, 34]. In laboratories, culture-based microbiological tests are widely used to determine the bacterial strains that are causing the invasion. The uterine cavity of cows has a distinct microbiome during gestation, even when the cervical plug is present and separates the uterine microbiota from the vaginal microbiota [35].

Recent studies have also showed that there are many other factors affecting the vaginal microbiota of cattle. For instance, figure 1 shows that there are at least 6 factors affecting the vaginal microbiota of cows like diet, age, weaning, feed supplements and most importantly antibiotics usage.

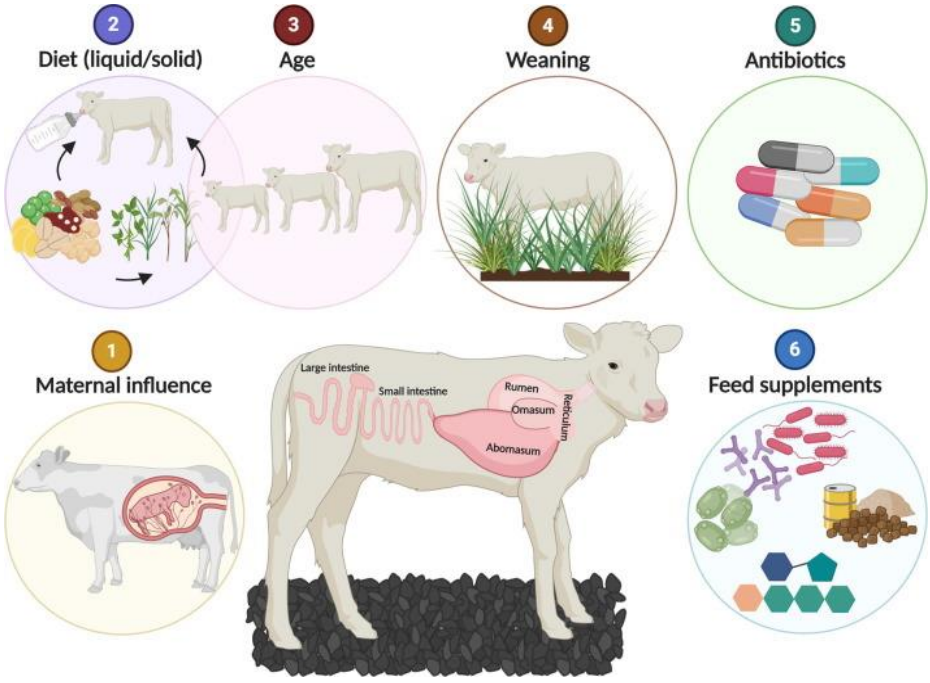


Figure 1: Factors affecting the Vaginal Microbiota of cows by Amin et al. (2021) [36]

2.5. Factors affecting the diversity of the genital microbiome

Animal genital tract microbial communities are spontaneously chosen due to their symbiotic roles. For instance, *lactobacilli* cling to the vaginal mucosa in females by using their tiny membrane projections, called fimbriae [37]. A useful supplier of nutrients for *Aggregatibacter spp*. is the collagen-rich tissue of the vagina. It is worthwhile to know that opportunistic microorganisms like *Histophilus*. In addition, there are additional variables that influence the variety of the genital microbes, some of which are intrinsic, like diet, and others which are extrinsic, like the phase of the female reproductive cycle. Due to striking parallels between the bacterial communities of the 2 biological sections, it is intriguing to speculate that the vaginal microbiota may have evolved from the gut microbiome [38].

2.6. Postnatal illnesses

With all species, vaginal childbirth poses a significant risk to the mom and her young, and cows are no different. Along with the dangers of physical harm during labor or inability to remove the placenta after delivery, the cow is frequently more susceptible to microbiological illnesses. The bacterial contamination of the uterus lumen following parturition has the most effect on health and production. Dairy cows raised in intensive farming settings, are animals that frequently develop microbial infection of the uterus. Indeed, the uterine cavity of 80–100% of mammals contains microorganisms in the first two weeks after calving. [39].

The involution of the uterus, endometrial renewal, the removal of microbial infection of the uterus, and the resumption of the ovary cycle activity are the processes that must be finished postpartum before a calf is likely to become pregnant once more. The evacuation of the fetus after calving, together with the accompanying tissues and fluids, serves as the first trigger for these alterations to take place. Numerous aerobic and anaerobic microorganisms can develop in the postpartum uterus canal environment. Numerous uterine defense systems eliminate most of these bacteria, which are pollutants in the uterine lumen. Nevertheless, *Escherichia coli*, *Arcanobacterium pyogenes*, *Fusobacterium necrophorum*, and *Prevotella species* are frequently linked to uterine illness [40]. The two pathogens with the highest prevalence rates in terms of numbers are *A. pyogenes* (49 %) and *E. coli* (37 % of pathogenic bacteria isolated) [41].

Postpartum, steroid hormone levels return to their baseline levels, and days following lactation, there is a rise in blood follicle stimulating hormone (FSH) level, which prompts the first post birth follicular phase to appear. Around 10–12 days following birthing, the initial dominant follicular sac is then chosen [42]. All maternity cows have these symptoms, regardless of periparturient illness, environmental factors, or nutritional limitations. It is commonly believed that an early resumption of menstrual cyclic action is advantageous for future fertility [43].

Healthy placenta ejection occurs in six hours following child discharge; however, if the placenta remains intact beyond 24 hours, it is referred to as a trapped placenta. In a farm, the prevalence of retained placenta ranges from 2 to 5 %, although it can rise in cattle having twins, following dystocia, and in areas with an epidemic transmission of communicable diseases. During first 2 weeks following birth, 25–40% of animals usually display medical metritis, and close to 20% of animals go on to develop clinical endometritis as a result of the condition.

Even though the clinical symptoms of endometrial illness, such as purulent discharge into the vagina, are easy to spot, the significance of preclinical uterus infection is poorly understood but is a growing problem. Neutrophils were found in the uterus canal or endometrial lining of up to 50% of calves 40–60 days following birth, along with inflammation of the organs; subclinical endometritis lowers the likelihood of conceiving [44].

Sheldon *et al*. have studied and outlined the classifications of uterus illnesses that affect cows (2006) [45]. Puerperal metritis is characterized by an unusually swollen uterus, a putrid wet red-brown uterus effluent, evidence of systemic sickness (reduced milk production, tiredness, or other toxaemia-related symptoms), and a temperature of greater than 39.5 °C in twenty-one days following parturition. Clinical metritis could be present in an animal in 21 days of birth if it has an unusually large uterus and a pus uterus secretion that can be seen in the vagina but is otherwise healthy. Purulent (>50 % pus) or mucopurulent (roughly 50 % pus, 50 % mucus) uterus output that is detected inside the genitals after 21 days of parturition, or both, are indicative of clinical endometritis. A calf is considered to have subclinical uterine infection if, uterine cytology tests conducted 21–33 days following birth contain more than 18% neutrophils or more than 10% neutrophils at 34–47 days. Pyometra is described as the buildup of pus matter in the uterus when there is a blocked cervix and a persisting corpus luteum.

2.7. Resultings from uterine illness

Infertility and partial fertility are linked to subclinical and clinical uterine disorders. This is described as longer times between birth and the initial fertilization or pregnancy of afflicted animals at the group level, as well as an increase in the number of cattle slaughtered for failing to reproduce at the proper time [46].

Following parturition, microbial infection of the uterus lumen commonly compromises reproductive activity in cows, leading to uterine illness, a major contributor to infertility [12]. Even though several cattle get rid of these germs during the first five weeks after giving birth, in 10–17 % of animals, infection persists and results in uterus illness that may be seen by medical assessment [47, 48]. Inflammatory response, histological endometrial lesions, a lag in uterus molting, and problems with embryo viability are all brought on by the existence of bacterial infections in the uterus.

2.8. Treatment possibilities

Recently, there has been a lot of research on using nonsteroidal anti-inflammatory medicines (NSAID) following birthing to enhance the welfare and productivity of dairy cows. Although various authors [49] found rather comparable findings about milk output in cows given with these medications post calving, their impacts on illness blockage and fertility are less uniform. For instance, retained fetal membranes (RFM) and metritis were more common in calves administered with flunixin meglumine directly following calving and 24 hours later. Nonsteroidal anti-inflammatory medicines (NSAIDs) function by preventing the creation of prostaglandins, which is crucial to the cascade of pathways that lead to vaginal delivery and the ejection of the fetus tissues [50]. As a result, treatment of strong NSAIDs within or right after childbirth could lead to significantly lower prostaglandin production, which might result in RFM and elevated incidence of other uterine illnesses including CM.

A mild NSAID (acetylsalicylic acid) was evaluated by other scientists for its impact on cattle health, but no change in illness frequency between control and treated animals was discovered [51]. Dairy farmers in the United States cannot use acetylsalicylic acid and its derivatives on breastfeeding calves since the FDA has not authorized them. CM is a common ailment in postnatal cattle that not only impacts the productivity and fitness of the cows but also their wellbeing [52]. According to Pascottini et al. (2020), postpartum heifers treated with an NSAID (meloxicam, for example) had enhanced neutrophil activity and their HP levels reduced from the second to the fourth day of therapy [53].

2.9. Resistance

One of the biggest concerns to people's health is antimicrobial resistance, or AMR. According to the Infectious Diseases Society of America *et al.* (2011), Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for the death of more Americans each year than emphysema, *HIV/AIDS*, Parkinson's disease kill all together [54]. All of the now available drugs are no, longer functional against these multidrug-resistant Gram-negative bacteria, especially those that produce carbapenemases, and there has been a startling dearth of discovery of new medications

active against them. The expense of health goes up as a result of resistance to antibiotics. According to Oxford and Kozlov (2013), the projected yearly cost of antibiotic resistance-related consequences in Europe is €9 billion. According to a recent assessment, the extra expense of resistance may amount to £20,000 for each hospital episode involving a patient.

The World Health Organization (WHO) envisions a post-antibiotic era in which there are little novel or new treatments in the drug research pipeline, and it issues a dire warning that could lead to harmless infections becoming possibly lethal as well as eradicating the medical advancements of the recent 100 years that have extended life in the majority of developing and developed countries. The war versus contagious diseases is being lost, and antibiotics could no longer be efficient later on, according to the Chief Medical Officer of the United Kingdom [55,56], who emphasized the need for physicians to maintain the efficacy of antibiotics by providing clear proof and guidance on their suitable use. Antibiotic use is crucial in other industries as well. For example, planting, farming, and fisheries account for around 80% of antimicrobial use in the US [57]. Studies indicate a clear link between antibiotic use and resistant of targeted bacteria. Resistance to antibiotics is more prevalent in nations with increased antibiotic usage.

Overmedication of antibiotics has been demonstrated to encourage patient coming back to the hospital because it medicalizes self-limiting diseases [58]. Higher attendance results even in greater antibiotic prescriptions. Therefore, the development of resistance by pathogenic strains may jeopardize not only the cure of contagious illnesses but also the use of a number of therapeutic procedures that are now assumed to be common procedure under the assumption that there are effective anti-drugs [59].

2.10. Antibiotic resistance mechanisms

An antibiotic's ability to limit growth of bacteria depends on how well it engages with its intended target. There are only these two factors that matter for this connection to take place: The destination must be recognized by the drug, and there must be sufficient amount present at the destination to effectively impede its function. Therefore, all resistance mechanisms either involve altering the receptor or lowering the amount of free antimicrobial drug that can reach it [60]. Resistance may result from mutations in the carrier, receptor, or enzyme genes that activate the pre-antibiotic. These processes are referred to as "passive mechanisms of resistance" since they

have no effect on the acting antibiotic itself. Apart from topoisomerase alterations in *Streptococcus pneumoniae* [61, 62] transmission of mutant genes through HGT typically does not impart sensitivity, which suggests that clonal expansion is the primary factor promoting the propagation of mutation-acquired antimicrobial resistance.

The mechanisms for resistance's spread and their genesis are thus crucial questions. Both mutations and horizontal gene transfer (HGT) can result in the establishment of resistant strains, which can lead to the development of bacterial resistance [63]. The body's flora or commensal (non-pathogenic) organisms may be the source of genes resistant to antibiotics. This suggests that these habitats need to be considered carefully if we wish to fully comprehend the process of acquiring and dissemination of antibiotic resistance among bacterial diseases.

Adding to these processes, tolerance can also be attained by lowering the quantity of antibiotic that is effective, either via alteration by enzymes that inactivate antibiotics or by export through multi-drug efflux systems. These components may be thought of as "active mechanisms of resistance," and when they are introduced into a new host, they can acquire resistance. This sort of tolerance can therefore propagate either by clonal expansion or through HGT [4].

We must take into account how microbial pathogenicity developed before the widespread use of medicines in the surroundings in order to understand what to anticipate in the coming days. The uncontrolled use of these medications has had a significant impact on the emergence of virulence, which has likely gone irrevocably in a new path. It is challenging to think of pathogenicity formation and resistance factor development over time in pathogens as separate processes due to their substantial overlapping. The influence on the world's microbiota, including that of humans, must be emphasized. The widespread use of antibiotics [64] results in the relocation of the microbiota or its mutation (antibiotic-resistant mutants) through the introduction of external antibiotic-resistant microorganisms.

Furthermore, resistant bacteria are now much more prone to displace well-adapted or migrating strains that are more vulnerable to the introduction of drugs. These strains are poor adapters or poor colonists of new habitats [6].

3. Aim

The aim of this research is to identify the naturally occurring inhabitants of vaginal microbiota in cows and determination of their antibiotic sensitivity.

3.1. Research questions

A. What are the naturally occurring microorganisms of the vaginal microbiota?
B. What are possible factors affecting the vaginal microbiota composition and activity?
C. How does antibiotic usage and overdose change the vaginal microbiota composition?

One of the goals of this study is to identify which bacterial species present as a normal microbiota in healthy cow vagina. For this we have chosen the MALDI-TOF method.
In the last ten years the development of matrix-assisted laser time-of-flight mass spectrometry, often known as MALDI-TOF MS, has become a cutting-edge approach for identifying a variety of species of bacteria. MS produced by MALDI-TOF MS typically measure the proteins in the form of ribosomes or peptides found in a pure sample. Because these proteins are so specialized for a particular bacterium, mass spectra may be thought of as species specific indicator, enabling precise genus- and identification of pure isolates [65]. The first stage in the examination of a specimen is the isolation of a group of isolates under various growing conditions, proceeded by a filtration process in which biomass is magnified to an adequate level [66]. Bacterial colonies are put onto a steel plate after culture, which takes up the majority of the analysis's duration. They are then covered with or combined with a suitable organic matrices mixture. The unlabeled spectrum of a new strain is detected utilizing computer software as a final step.

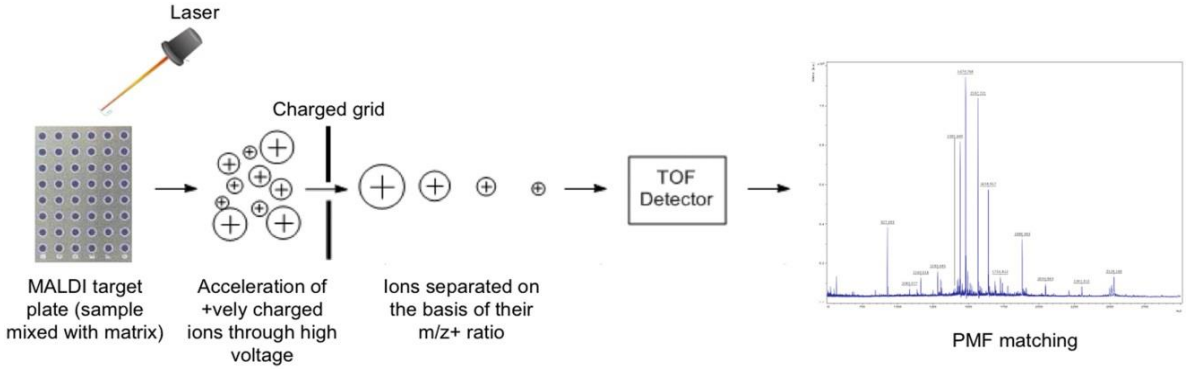
Our further aim is to test the antimicrobial susceptibility of the members of normal vaginal microbial communities, if they are sensitive, intermediate, or resistant to different antibiotics. For this we choose the broth microdilution method.

4. Materials and Methods

In our study we determined the most frequent inhabitant bacteria from the vagina of 67 healthy dairy cows prior to the parturition and after. The breed of cows was Holstein Friesian, the animals were allocated in three different dairy farm units. Samplings were performed with the consent of the animal welfare representative of each farm unit. Sterile phosphate-saline buffer (100 ml/cow) was used for vaginal rinsing. Before flushing step, the pH was determined with contact pH measuring instrument. The isolated bacterial species were identified with MALDI-TOF method.

4.1. MALDI-TOF method

The pure cultures of isolated bacteria were analysed by MALDI-TOF method (Figure 2). Identification of microorganisms by MALDI-TOF MS is carried out by correlating the PMF of unknown sample with the PMFs present in the dataset. When performing PMF matching, unknown microbial isolates' MS spectra are compared to those of known microbial isolates that are stored in the database. Microbes are identified at the species level using a mass range m/z of 2–20 kDa, which mostly reflects ribosome subunits and a few maintenance proteins. By comparing a specific microorganism's PMF sequence to the PMFs of the ribosomal proteins in a large accessible dataset, it is possible to recognize the microbe. Abundant rRNA proteins which make up about 60–70% of the mass of a microbe have a distinctive pattern. At the end of the analyses the microorganisms are determined at the genus or at the species level.



<u>Figure 2:</u> This figure shows the priciple of MALDI-TOF method. [67]

4.2. Genomic DNA isolation and PCR analysis

Five bacterial isolates were selected for further examinations, including a *Brevibacillus agri, Bacillus pumilus, Bacillus subtilis*, and two *Bacillus licheniformis* strains. Bacterial broth cultures were grown for one day at 37 °C. Each 10 ml bacterial culture was centrifuged for 10 minutes at 3000 g. After that, 500 µl of lysis solution was added to the cell pellet and then the cells were resuspended by gentle vortexing. The mixture was pipetted into 2 ml tube, containing 0.1 mm diameter beads and the tubes were placed horizontally on a flat-bed vortex pad and were vortexed for 20 minutes at maximum speed. The lysate-containing tubes were then incubated at 65°C for 10 min, after this the lysate was transferred to a clean microcentrifuge tube and centrifuged at 14,000 g for 2 min. The clean supernatant (150 µl) was pipetted to a clean tube and first 150 µl ethanol, then 250 µl binding solution was added, and the tubes were thoroughly vortexed. The following steps were done using the Reliaprep^{TM} gDNA miniprep system (Promega) according to the manufacturer's instructions.

PCR analysis was applied for the taxonomical identification of bacteria and for the detection of selected resistance genes. PCR was performed using Firepol PCR supermix (Solis Biodyne) on the CFX Opus Real-Time PCR System (Bio-Rad). For each PCR reaction, 2 µl 2 ng/µl DNA was added directly to a PCR reaction mixture, in a final volume of 20 µl, containing nuclease-free water, 4 µl PCR supermix and 0.2 µM of the forward and reverse primers. The thermal profile for all reactions was 12 min at 95°C, then 30 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C.

4.3. Microplate broth dilution method

The CLSI (2015) certified broth microdilution technique was applied. The 96-well sterile microplates with flat bottom were employed for the assays.

By the use of a multichannel micropipette first, 100 µl brain heart infusion (BHI) broth containing the antimicrobial substances in serial dilution was added to each well of the microplate. The tested antimicrobial substances were; amoxicillin trihydrate, ceftiofur hydrochloride, cefquinome sulfate, oxytetracycline hydrochloride, doxycycline hyclate, sulphametoxazole, trimethoprim, florfenicol, marbofloxacin, tylosin tartrate, and tulathromycin. To the control wells 100 µl BHI was added. Secondly, the inoculation of bacteria was made in a final concentration of 10^4 CFU per well. The

dishes were cultivated for 24 hours at 37 °C. After that, the absorbance of each plate was measured at 600 nm to detect any level of bacterial growth (Figure 3).

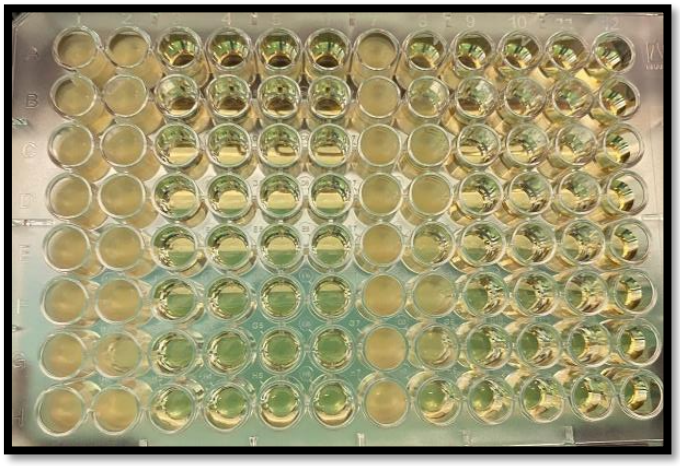


Figure 3: Broth microdilution test on 96-well microplate

5. Results

The cows used for vaginal sampling did not show vaginal discharge and the average pH of their vaginal mucosal surface was 7.27±0.15.

5.1. MALDI-TOF results

The identification of the species of bacteria present as a normal flora in the cow's vaginas was done by the use of the MALDI-TOF MS experiment as described in the methods part. The results of this experiment are illustrated in *table 1* below.

As can be seen from the *table 1*, the majority of the normal microbiota of the cow's vagina constitute of Gram-positive bacteria where 47 species were detected. The minority of the bacteria are Gram-negative with only 7 identified species.

Table 1: The different bacterial species present in the vaginal microbiota in healthy cows.

Gram-positive	Gram-negative
Arthrobacter gandavensis	*Acinetobacter pittii*
Bacillus amyloliquefaciens	*Actinobacillus rossii*
Bacillus cereus	*Bacteroides fragilis*
Bacillus circulans	*Campylobacter hyointestinalis*
Bacillus clausii	*Escherichia coli*
Bacillus licheniformis	*Mannheimia varigena/haemolytica/granulomatis*
Bacillus megaterium	*Proteus mirabilis*
Bacillus oceanisediminis	
Bacillus oleronius	
Bacillus pumilus	
Bacillus safensis	
Bacillus siralis	
Bacillus sonorensis	
Bacillus subtilis	
Bifidobacterium pseudolongum	
Brevibacillus agri	
Brevibacillus borstelensis	
Brevibacillus parabrevis/agri	
Corynebacterium camporealensis	

Table 1 (continued)	
Corynebacterium renale	
Corynebacterium xerosis	
Enterococcus avium	
Enterococcus hirae	
Lysinibacillus fusiformis	
Lysinibacillus massiliensis	
Micrococcus luteus	
Paenibacillus cookii	
Paenibacillus ihumii	
Paenibacillus lactis	
Peptoniphilus indolicus	
Staphylococcus chromogenes	
Staphylococcus epidermidis	
Staphylococcus hominis	
Staphylococcus succinus	
Staphylococcus sciuri	
Staphylococcus xylosus	
Streptococcus alactolyticus/lutetiensis	
Streptococcus canis	
Streptococcus dysgalactiae	
Streptococcus equinus	
Streptococcus lutetiensis	
Streptococcus mitis/oralis/peroris	
Streptococcus pneumoniae/pseudopneumoniae	
Streptococcus pluranimalium/hyovaginalis	
Streptococcus suis	
Streptococcus uberis	
Trueperella pyogenes	

The frequency of each genus of the identified bacteria is represented in the graph *(figure 4)* below. As illustrated, the highest frequency of bacterial genus was the *Bacillus genus* with 13 out of the 47 Gram-positive bacteria identifies. This is followed by *Streptococcus genus* that takes 10 of 47 of the identified Gram-positive bacteria. *Staphylococcus* comes next with 6 out of 47, then *Brevibacillus*, *Corynebacterium*, *Paenibacillus* with 3 out of 10. For the rest of the Gram-positive and the Gram-negative identified genus, there were only one detected species for each.

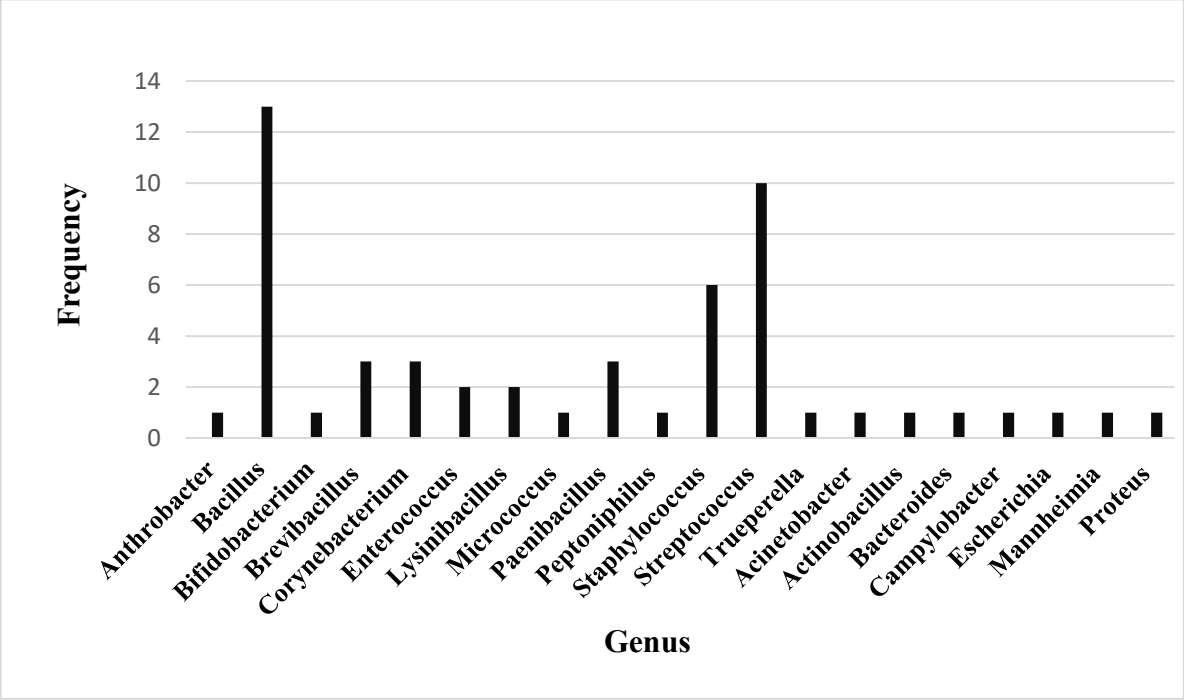


Figure 4: Frequency of each bacterial genus identified in the vaginal microbiota of the cows.

5.2. PCR results

The taxonomic species of the five selected bacterial isolates was confirmed by PCR *(table 2)*. The genome of *Brevibacillus agri* and one of the two *Bacillus licheniformis* isolates did not contain beta-lactamase resistance gene. Nonetheless, the genome of the other *B. licheniformis* and the *B. pumilus* and *B. subtilis* contained beta-lactamase resistance genes.

Table 2: Identified bacterial species and the presence of resistance genes.

Species confirmed by PCR	Resistance gene	Present
Brevibacillus agri	class A beta-lactamase	**No**
Bacillus licheniformis (W)	beta-lactamase PenP	**No**
Bacillus licheniformis	beta-lactamase PenP	**Yes**
Bacillus pumilus	beta-lactamase class D	**Yes**
Bacillus subtilis	beta-lactamase class A, PenP	**Yes**
	beta-lactamase class D, ybxI	**Yes**

5.3. Minimum Inhibitory Concentration results

The MIC of the five isolates to different antibiotics were detected by the use of a 96-well plate broth dilution method *(table 3)*. The MIC of sulphametoxazole-trimetoprim was 500 mg/l to *Bb. agri*, and the MIC of the other tested antibiotic substances were or below 2 mg/l. The MIC of amoxicillin was 50 mg/l to the *B. licheniformis*, and the MIC of the remaining agents were or under 4 mg/l. Regarding MIC of amoxicillin and cefquinome was above 5 mg/l and 8 mg/l to *B. licheniformis W* and it was 8 mg/l in case of ceftiofur. As the MIC of amoxicillin, cefquinome and ceftiofur was above 5 mg/l, 8 mg/l and 2 mg/l to *B. pumilus*, respectively. The MIC of the oxytetracycline was the highest in case of *B. subtilis* cultures while for florfenicol was between 1-4 mg/l to the tested bacteria. While the MIC of doxycycline, marbofloxacin, and tylosin was below 1 mg/l in all cases, and the highest MIC of tulathromycin was 2 mg/l.

Table 3: Minimum inhibitory concentration (MIC) of the tested antimicrobial substances.

	AMX	CTF	CFQ	OTC	DOX	SMZ TMP	FFC	MBF	TYL	TUL
	MIC (mg/l)									
Brevibacillus agri	0.5	0.016	0.06	1	0.05	500	2	0.5	0.5	0.5
Bacillus licheniformis	>50	0.5	1	2	0.5	1	4	0.125	0.5	0.5
Bacillus licheniformis (W)	>5	8	>8	0.5	>0.05	2	4	0.25	0.25	2
Bacillus pumilus	>5	>2	>8	0.5	0.05	>2	4	0.5	0.5	1
Bacillus subtilis	>50	0.25	0.25	8	0.5	1	1	0.25	0.5	2

AMX (amoxicillin) – CFT (ceftiofur) – CFQ (cefquinome) – OTC (oxytetracycline) – DOX (doxycycline) – SMZ TMP (sulphametoxazole-trimetoprim) – FFC (florfenicol) – MBF (marbofloxacin) – TYL (tylosin) – TUL (tulathromycin)

6. Discussion

The data from MALDI-TOF *(table 1)* shows that the vast majority of the normal microbiota are Gram-positive bacteria with 87% (47 out of 54) of them are Gram-positive. This should be the normal case which means the results are as expected. This is because Gram-positive bacteria are less pathogenic than Gram-negative bacteria because the latter has their LPS layer that is toxic to our cells and they can release more endotoxins that are more harmful than those produced by Gram-positive ones (WebMD, 2021) [68]. Therefore, it is evolutionary advantageous for the cows to have Gram-positive bacteria as their normal flora, so that when an abnormality occurs, and they transform to their virulent form (through transduction or other bacterial infection) their effects and pathogenicity would be less harmful and damaging than that of Gram-negative bacteria. These findings align with those of Nava *et al.* (2011) that shows that Gram-positive bacteria predominate over Gram-negative bacteria in the vaginal microbiota of Criollo Limonero cows with 81.82% being Gram-positive while only 18.18% were Gram-negative [69].

The high frequency of *Bacillus species* is highly beneficial for the cows' vaginal tract, because *Bacillus species* is considered as a probiotic bacterium and its spores are utilized as probiotics (Aly *et al*., 2008) [70]. This is of a high importance because the fact that it is sporulated means that highly stable and that it may be not harmed or killed by temporary changes in the vaginal microenvironment. These results coincide with the findings of the study conducted by Otero *et al*. (2000) that showed that *Enterococcus spp.*, *Staphylococcus spp.*, and *Streptococcus spp.* are the most common bacteria isolated from the cow's vaginal tracts [71].

Table 4: The susceptibility of the selected isolates against different antibiotics.

Substance	*Brevibacillus agri*	*Bacillus licheniformis*	*Bacillus licheniformis (W)*	*Bacillus pumilus*	*Bacillus subtilis*
Amoxicillin	S	R	I	I	R
Cefquinome	S	S	R	R	S
Ceftiofur	S	S	I	I	S
Oxytetracycline	S	S	S	S	R
Doxycycline	S	S	S	S	S
Sulfamethoxazole-trimethoprim	R	S	S	I	S
Tylosin	S	S	S	S	S
Tulathromycin	S	S	S	S	S
Florfenicol	S	S	S	S	S
Marbofloxacin	S	S	S	S	S

S (sensitive), I (intermediate), R (resistant)

(Breakpoints were extrapolated according to available values for *B. anthracis* or *B. cereus*)

The antimicrobial susceptibility of the tested bacteria based on the gained MIC values is represented in *table 4*. The *Brevibacillus agri* isolate showed resistance against sulfamethoxazole-trimethoprim (MIC is 500 mg/l) and sensitiveness towards the rest of the antibiotics. One of the *Bacillus licheniformis* isolate showed resistance only against amoxicillin (MIC is >50 mg/l), while was sensitive to the rest of the used antibiotics. The *Bacillus licheniformis W* isolate showed resistance against cefquinome only (MIC > 8 mg/l); and showed intermediate resistance against amoxicillin (MIC is >5 mg/l), and ceftiofur (MIC is 8 mg/l), however was sensitive to the rest of the antibiotics. The *Bacillus pumilus* showed resistance against cefquinome (MIC is > 8 mg/l); and showed intermediate resistance against ceftiofur (MIC is >2 mg/l), amoxicillin (MIC is >5 mg/l), and sulfamethoxazole-trimethoprim (MIC is >2 mg/l), and was sensitive to the rest of the antibiotics. The *Bacillus subtilis* isolate was resistance to oxytetracycline (MIC is 8 mg/l) and amoxicillin (MIC is >50 mg/l), and was sensitive to the rest.

These results showed that the studied bacterial species mainly *Bacillus genus* showed sensitiveness or intermediate resistance on almost all the used antibiotics. This indicates that these strains can be highly affected by the abnormal and unplanned antibiotic use, which may lead to the death of this microbiota and as a result dysregulate the microenvironment like the pH and certain ionic and molecular concentrations. This would have several side effects that can impact fertility, cause infections, or any other reproductive disorder. On the other hand, these results coincide with

the results provide by Jetres *et al.* (2009) in their study that showed that increasing the antibiotic uptake has significantly decreased the normal microbiota in cows [72].

Furthermore, an increased MIC was noticed in the case of amoxicillin, which might be explained by the presence and expression of beta-lactamase; although the *B. pumilus* and *B. licheniformis* bacterium strains showed contrastingly a sensitivity to the cephalosporins used in the study (cefquinome and ceftiofur). In fact, the study from Bucher *et al.* (2019) established a connection between the transcriptional activation of beta-lactamase, due to generic bacterial cell wall stress, and the resistance to the ensuing exposure to penicillins – especially ampicillin [73].

In conclusion, our study demonstrated that the majority of the normal vaginal microbiota of cows is composed of Gram-positive bacteria in which *Bacillus genus* was the most common followed by *Streptococcus* and then *Staphylococcus genus*. As our results showed that the tested bacterial isolates were sensitive or had intermediate resistance to most of the tested antibiotics. For future knowledge and advancement on this topic further studies such as susceptibility tests should be done for additional clarifications, as well as other experiments on more sensitive bacteria that could potentially be safe to use in animals.

7. Abstract

Microbiota in the bodies of different species have a significant impact on the health status of the host. Most studies have focused on the microbiota of gut, skin, and mouth, and not too much research known about normal vaginal microbiota in livestock. The characteristic bovine uterine diseases such as metritis and endometritis can be the results of colonization through the extrinsic and ascending pathways to the vagina, rarely the intestinal bacteria may contaminate the uterus.

In our studies we determined the most frequent inhabitant bacteria from the vagina of healthy dairy cows prior to the parturition and after. Sterile phosphate-saline buffer (100 ml/cow) was used for vaginal rinsing. Before flushing step, the pH was determined with contact pH measuring instrument. The bacteria were cultivated from the rinsing liquid using four different culturing media under aerobic and anaerobic conditions. The taxonomical identification of bacteria was determined by Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) method.

Among the frequently occurring non-pathogenic bacteria, five bacterial isolates were selected for further examinations, including *Brevibacillus agri, Bacillus pumilus, Bacillus subtilis*, and two *Bacillus licheniformis* strains. The species identification of bacteria was confirmed by polymerase chain reaction (PCR) analysis. The presence of known resistance genes was also examined by PCR. Broth microdilution susceptibility testing was performed using 10 different antimicrobial agents. The examined isolates were highly sensitive to tylosin, tulathromycin, doxycycline, and marbofloxacin. The presence of a beta-lactamase resistance gene was confirmed in the genome of three isolates.

With the knowledge of the normal vaginal microbiota of cows and knowing their resistance patterns more specific and efficient prevention of the peri/post-partum diseases may be possible.

8. Összefoglaló

A különböző fajok testében élő mikroorganizmusok jelentős mértékben befolyásolják a gazdaszervezet egészségi állapotát. A legtöbb tanulmány elsősorban a bélrendszer, a bőr és a száj mikrobiotáját vizsgálja, azonban kevesebb kutatás foglalkozik a gazdasági haszonállatok normál hüvelyi mikrobiotájának összetételével. A szarvasmarhák méhbetegségei jellemzően például a metritis és az endometritis, melyet a hüvelyből bejutó kolonizáló baktériumok okozhatnak, továbbá ritkábban a bélbaktériumok is bejuthatnak a méhbe.

Vizsgálataink során meghatároztuk az egészséges tejelő tehenek hüvelyéből származó leggyakrabban előforduló baktériumokat az ellés előtt és után. A hüvely átöblítéséhez tehenenként 100 ml steril foszfáttal pufferelt sóoldatot alkalmaztunk. Az átöblítést megelőzően minden egyed esetében megmértük a hüvely pH-ját. A hüvelymintákat négy különböző típusú tápközegre szélesztettük, a párhuzamos mintákat aerob és anaerob körülmények között inkubáltuk. Az izolált baktériumok rendszertani besorolásának beazonosítását mátrix-asszisztált lézer deszorpciós, ionizációs, repülési idő mérésén alapuló tömegspektrometria (MALDI-TOF) módszerrel végeztük.

A gyakran előforduló nem patogén baktériumok közül öt baktérium izolátumot választottunk ki a további vizsgálatokhoz; *Brevibacillus agri, Bacillus pumilus, Bacillus subtilis* és két *Bacillus licheniformis* törzset. A baktériumok fajának pontos beazonosítását polimeráz-láncreakció (PCR) analízissel végeztük, továbbá, ismert rezisztenciagéneket kerestünk a genomjukban. A baktériumok érzékenységét tíz antimikrobás hatóanyaggal szemben határoztuk meg, mikro-leveshígítás módszerrel. A vizsgált izolátumok mindegyike érzékeny volt tilozinra, tulatromicinre, doxiciklinre és marbofloxacinra. Három izolátum genomjában beta-laktamáz rezisztencia gén jelenlétét igazoltuk.

A tehenek normál hüvelyi mikrobiota összetételének részletesebb megismerése, valamint az antimikrobiális rezisztencia mintázatuk ismerete elősegítheti az ellés előtti és utáni betegségek hatékonyabb megelőzését és kezelését.

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Appendix 5. Declaration regarding TDK research paper-thesis equivalence

DECLARATION

I hereby declare that the thesis entitled ***"Identification of naturally occurring inhabitants of vaginal microbiota in cows and determination of their antibiotic sensitivity"***
is identical in terms of content and formal requirements to the TDK research paper submitted in 2022.

Date: 30th – October – 2023

Jad El Hawly

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