Araştırma Makalesi/Original Article
Makale Dili "İngilizce"/Article Language "English"

Türk Hijyen ve Deneysel Biyoloji Dergisi

# Evaluation of cultivable aerobic vaginal microbiota of repeat breeder and healthy pregnant dairy cows

## Tekrarlayan kızgınlık yaşayan ve sağlıklı gebe süt ineklerinin kültürü yapılabilir aerobik vajinal mikrobiyotasının değerlendirilmesi

Elçin GÜNAYDIN[1] (ID), Gülsen GONCAGÜL[2] (ID), Emsal Sinem ÖZDEMİR SALCI[3] (ID)


### ABSTRACT

**Objective:** Vaginal microbiota on bovine fertility has been minimally explored, and data on this are limited, despite the fact that vagina itself is a first barrier against ascending pathogens in the reproductive tract and interacts with host mucosa. Contamination of the female genital tract or dysbiosis of the vaginal flora have risks for the development of reproductive problems. Subclinical endometritis is one of the many variables that might lead to repeat breeder syndrome, and its significance has been neglected. In this study, we aimed to examine cultivable aerobic vaginal microbiota of repeat breeder (RB) cows and healthy pregranant (HP) cows at 60$^{th}$ day following artificial insemination.

**Methods:** A total of 45 Holstein breed dairy cows aged between 3 and 8 years including 20 repeat breeder (RB) cows (Group I = RBG), alongside 25 healthy pregnant (HP) cows (Group II=HPG) that conceived within one or two inseminations were used as study groups. The vaginal swab samples collected from RBG and HPG were incubated at 37 °C for 24 hours in fluid thioglycollate. On the next day, part of the initial culture was inoculated onto blood agar and EMB agar examined for

### ÖZET

**Amaç:** Vajinal mikrobiyotanın sığır fertilitesi üzerindeki etkisi çok az araştırılmıştır ve bu konuda sınırlı veri mevcuttur. Vajinanın kendisi üreme yolunda yukarı doğru çıkan patojenlere karşı ilk bariyer olup konak mukozası ile etkileşime girer. Dişi genital yolun kontaminasyonu veya vajinal floranın disbiyozu, üreme problemlerinin gelişmesi için risk taşır. Subklinik endometrit, tekrarlayan kızgınlık sendromuna yol açabilecek birçok değişkenden biridir ve önemi göz ardı edilmiştir. Bu çalışmada, yapay tohumlamadan sonraki 60. günde tekrarlayan kızgınlık (RB) inekleri ve sağlıklı gebe (HP) ineklerin kültürü yapılabilir aerobik vajinal mikrobiyotasını incelemeyi amaçladık.

**Yöntem:** Çalışma grupları olarak, 3 ila 8 yaşları arasında 20 tekrarlayan kızgınlık (RB) ineği (Grup I = RBG) ve bir veya iki tohumlama içinde gebe kalan 25 sağlıklı gebe (HP) ineği (Grup II=HPG) olmak üzere toplam 45 Holstein cinsi süt ineği kullanıldı. RBG ve HPG'den alınan vajinal sürüntü örnekleri, sıvı tiyoglikolat içinde 37 °C'de 24 saat inkübe edildi. Ertesi gün, başlangıç kültürünün bir kısmı kanlı agar ve EMB agar üzerine ekilerek kültürlenebilir vajinal mikrobiyota


[1]Kastamonu University, Veterinary Faculty, Department of Microbiology, Kastamonu, Türkiye
[2]Bursa Uludag University, Mennan Pasinli Equine Vocational School, Bursa, Türkiye
[3]Bursa Uludag University, Veterinary Faculty, Department of Obstetrics and Gynecology, Bursa, Türkiye

İletişim / Corresponding Author : Elçin GÜNAYDIN
Kastamonu Üniversitesi Veteriner Fakültesi Kastamonu - Türkiye
E-posta / E-mail : elcin_gunaydin@hotmail.com

Geliş Tarihi / Received : 21.06.2024
Kabul Tarihi / Accepted : 22.07.2024

DOI ID : 10.5505/TurkHijyen.2024.13914

Günaydın E, Goncagül G, Özdemir Salcı ES. Evaluation of cultivable aerobic vaginal microbiota of repeat breeder and healthy pregnant dairy cows. Turk Hij Den Biyol Derg, 2024; 81(3): 329 - 338

the cultivable vaginal microbiota. The identification of bacteria was done with an automated system.

**Results:** A total of 26 species of 16 genera distributed among 4 phyla including Proteobacteria, Firmicutes, Actinobacteria, Bacteroiodetes were identified. The relative abundance of Proteobacteria in HP group comparing with the RB group was associated with an increase in progesterone. HP group presented relatively greater richness with 21 species followed by the RB group with 17 identified species. HP group possessed a more diverse microbiota in comparison to those suffering from subclinical endometritis. The relative abundance of *Escherichia* genus and *Escherichia coli* in RB group might have induced supressing the release of luteinizing hormone.

**Conclusion:** As a conclusion, the diversity of the vaginal microbiota and numerically abundance of some genus and species had favourable/unfavorable effects on reproductive fertility. The consortium of vaginal microbiota should be explored entirely in further studies since vagina is the first entrance of pathogens via ascending route in the reproductive tracts.

**Key Words:** Repeat breeder, pregnant, cows, vaginal microbiota

için incelendi. Bakterilerin tanımlanması otomatize bir sistem ile gerçekleştirildi.

**Bulgular:** Proteobacteria, Firmicutes, Actinobacteria ve Bacteroidetes dahil olmak üzere 4 şubeye dağılan 16 cinse ait toplam 26 tür tespit edilmiştir. HP grubunda Proteobacteria'nın relatif bolluğunun RB grubuna kıyasla progesteron artışı ile ilişkili olduğu bulunmuştur. HP grubu, 21 tür ile nispeten daha büyük bir zenginlik göstermiş, bunu 17 tür ile RB grubu takip etmiştir. HP grubu, subklinik endometritis yaşayanlara kıyasla daha çeşitli bir mikrobiyotaya sahipti. RB grubunda *Escherichia* cinsi ve *Escherichia coli*'nin relatif bolluğu, luteinize edici hormonun salınımını baskılamış olabilir.

**Sonuç:** Sonuç olarak, vajinal mikrobiyotanın çeşitliliği ve bazı cins ve türlerin sayısal bolluğu üreme fertilitesi üzerinde olumlu/olumsuz etkilere sahiptir. Vajina, üreme yolunda patojenlerin yukarı doğru girişi için ilk giriş noktası olduğundan vajinal mikrobiyota konsorsiyumu gelecekteki çalışmalarda ayrıntılı araştırılmalıdır.

**Anahtar Kelimeler:** Tekrarlayan kızgınlık, gebe, inekler, vajinal mikrobiyota

## INTRODUCTION

The prospective origin of the reproductive tract microbiota is diverse. Microorganisms can enter the reproductive tract from other anatomical sites. In particularly, weakened physical cervical barrier at birth allows the introduction of bacteria into the genital system either from the vagina or from the environment via the vagina and also from the feces and animal skin to the genital tract (1). Besides this, negative abdominal pressure after parturition which causes a craniad sinking of the anus and subsequently of the vagina with increased craniad angulation of the dorsal portion of the vulva in predisposed animals. Air sucking in severely affected animals additionally triggers a reflux of urine from the vestibule to the vagina which results in urine accumulation in the lower part of the vagina (pneumovagina) (2). The cattle rumen typical flora such as *Porphyromonas*, *Fusobacterium*, and *Bacteroides* (3) were excreted in feces and then because of enviromental contamination uterus infections increased, subsequently occurence of metritis (4). Moreover, vaginal flora and uterus flora shares the same pathogens as previously described (5). Plausible pathogenicity of bacterial categories isolated from the lumen of the uterus are divided into three categories: Uterine pathogens, *Trueperella*, *Escherichia coli*, *Prevotella* spp., *Bacteroides* spp.,

*Fusobacterium* spp.; Potential pathogens, *Bacillus licheniformis, Enterecoccus faecalis, Pasteurella* spp., *Staphylococcus aureus, Peptostreptecoccus* spp., *Nonhaemolytic streptecocci*; Oportunistic contaminants, *Clostridium perfringens, Klebsiella pneumonia, Micrococcus* spp., *Proteus* spp., *Aspergillus* spp., *Streptecoccus* spp., *Haemolytic streptecocci* (6). The ascension pathway of bacteria into the uterus is likewise plausible from vagina. Additionally, all of these bacteria are the result of fecal contamination of bedding, the environment, and fur (7).

In cattle breeding, reproductive efficiency is the most important criterion of productivity. Reproductive yield is reduced due to genetic, nutritional, hormonal or infectious problems. (8-10). In these reproductive problems, reproductive canal infections affect fertility and cause economic loss (10). Changes in vaginal microflora may lead to infertility in cattle (11). Since the host microorganisms of the vaginal flora are in permanent interaction with the mucosa they are the first protective barrier against the pathogens and sperm carried by the ascending route in the genital tract (11). On the other hand, presence of specific bacterial populations in the vagina has risks for the development of reproductive problems (11,12). Contamination of the female genital tract or dysbiosis of the vaginal flora leads to infections of genital tract, abortion, premature parturition (10,11). Repeat breeding syndrome is another major infertility problems of herds (13). Animals exhibiting regular estrus cycle and normal heat signs but failed to conceive after three or more inseminations are named as repeat breeders (13). Subclinical endometritis is one of the many variables that might lead to repeat breeder syndrome, and its significance shouldn't be overlooked.

Although studies on genital canal flora in cattle generally focus on uterus. Considering the significance of vaginal flora being the first entrance of microorganisms to genital canal, this study aimed to compare the vaginal cultivable aerobic bacterial flora of repeat breeder cows and healthy pregnant cows in order to discuss the effects on reproduction in the light of literatures.

## MATERIAL and METHOD

### Study groups

A total of 45 Holstein breed dairy cows aged between 3 and 8 years including 20 repeat breeder (RB) cows (Group I = RBG) that have undergone three or more inseminations without conception and showed no genital pathology, alongside 25 healthy pregnant (HP) cows (Group II=HPG) that conceived within one or two inseminations were used as study groups

### Sampling

The vaginal swab samples collected from RBG and HPG were collected on the 60th day following artificial insemination. Before vaginal swab sampling from cows in order to prevent contamination, the tail was lifted upwards, and the external genital area with vulval lips was cleaned with benzalkonium hydrochloride (Zefirolum®, Kimpa, Istanbul). Then, the area was dried with a sterile towel. Subsequently, with gloved hands, a sterile cotton swab on a polypropylene shaft was rotated for more than 10 seconds between the opened vulval lips (by the same individual each time), sampling was done from the posterior and dorsal aspects of the vagina. Swabs were transferred into sterile tubes containing thioglycolate broth as a transport media and transported to the laboratory at 4 °C and immediately processed for bacteriological examination.

### Bacteriological examination

For bacteriological examination, all vaginal swab samples were incubated at 37 °C for 24 hours in fluid thiogly-collate (Becton-Dickonson BBL, 221196, USA). On the next day, part of the initial culture was inoculated onto blood agar (Becton-Dickonson BBL, 297876, USA ) and EMB agar (Eosin-Methylenblue-Lactose-Saccharose) (Becton-Dickonson BBL, 221355, USA) and incubated at 37 °C for another 24 hours. According

to colony morphology and Gram color features, identified colonies were quantitatively assessed. For the identification of the bacteria, their direct cultures were performed using BBL Crystal (Becton-Dickinson, Sparks, USA) Gram-Positive and Gram-Negative ID system kits and its computer program.

According to the subparagraph k of the 8th article of the "Regulation on the Principles and Procedures of Animal Experiment Ethics Committees" published in the Official Gazette dated 15.02.2014 and numbered 28914, the collection of fecal or bedding samples and sample collection by swabbing are not subject to the approval of the Local Ethics Committee for Animal Experiments (HAYDEK).

## RESULTS

### Creating the OA Model

Frequency of 45 samples with bacterial isolation was determined to be 86.66% (39/45). All of the vaginal swab samples (100%) collected from RBG exhibited bacterial growth whereas 19 out of 25 vaginal swabs (76%) from HPG were found to be positive in terms of bacterial isolation.

A total of 26 species of 16 genera distributed among 4 phyla including Protobacteria, Firmicutes, Actinobacteria, Bacteriodetes were identified (Figure 1). Comparing the number of phyla distributed between two groups were as follows: Twenty-one Proteobacteria, three Actinobacteria were determined in RBG. One Bacteriodetes, 32 Protobacteria, two Actinobacteria were detected in HPG. Both groups shared the same number of bacteria (n=14) belonging to Firmicutes phylum (Figure 1). The most frequent species belonging to predominant genera for RBG and HPG were *Bacillus* (36.84%, n=14/38), *Sphingomonas* (15.78%, n=6/38), *Flavimonas* (n=13.15%, 5/38), *Esherichia* (7.89%, n=3/38) *Corynebacterium* (7.89%, n=3/38) genera, and *Bacillus* (31.25%, n=15/48), *Sphingomonas* (18.75, n=9/48), *Flavimonas* (14.5%, 7/48), *Actinobacter* (6.25, n=3/48) genera, respectively (Figure 2).

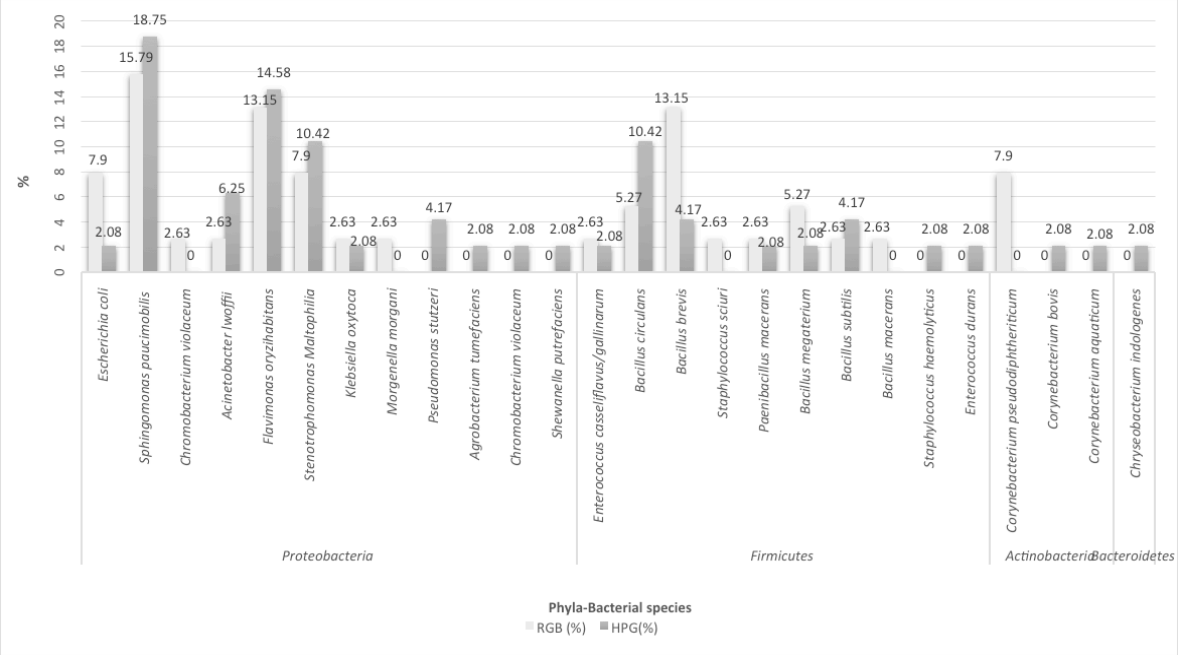


**Figure 1.** Distribution of species and phyla between Repeat Breeder Group (RBG) and Healthy Pregnant Group (HPG)

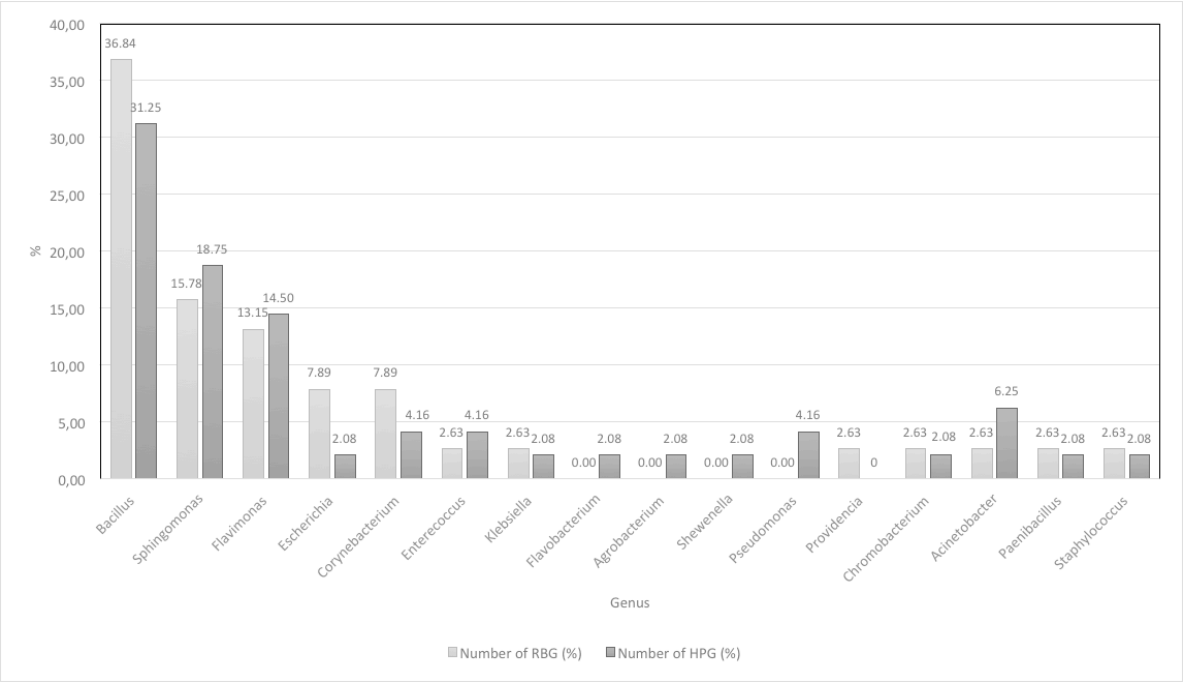


**Figure 2.** Distribution of genera between repeat breeder group (RBG) and healthy pregnant group (HPG)

In RBG, the most isolated species shared the first five places were Sphingomonas paucimobilis (15.79%), *Flavimonas oryzihabitans* (13.15%), *Bacillus brevis* (13.15%), *Escherichia coli* (7.90%), *Corynebacterium pseudodiphtheriticum* (7.90%), and *Stenotrophomonas maltophilia* (7.90%) while *Sphingomonas paucimobilis* (18.75%), *Flavimonas oryzihabitans* (14.58%), *Stenotrophomonas maltophilia* (10.42%), *Bacillus circulans* (10.42%), and *Acinetobacter iwoffii* (6.25%) was observed as the first five species (Figure 1). Regarding cows with RB syndrome Bacillus represented most heteregenous group, including 6 different species with a predominance of *Bacillus brevis* (13.15%) while HPG represented 5 different species (Figure 1).

HPG presented relatively numerically greater richness with 21 species followed by the RBG group with 17 identified species. Bacterial species common to both two groups were as follows: *E. coli*, *Enterococcus casseliflavus/gallinarum*, *Sphingomonas paucimobilis*, *Bacillus circulans*, *Bacillus brevis*, *Acinetobacter iwoffii*, *Flavimonas oryzihabitans*, *Paenibacillus macerans*, *Bacillus megaterium*, *Stenotrophomonas maltophilia*, *Bacillus subtilis*, *Klebsiella oxytoca* (Figure 1). The following were the bacterial species that were isolated in solely RBG: *Chromobacterium violaceum*, *Staphylococcus sciuri*, *Corynebacterium pseudodiphtheriticum*, *Bacillus macerans*, and *Morgenella morgani*. *Agrobacterium tumefaciens*, *Staphylococcus haemolyticus*, *Chryseobacterium indologenes*, *Chromobacterium violaceum*, *Corynebacterium bovis*, *Shewanella putrefaciens*, *Enterococcus durans*, *Corynebacterium aquaticum* were determined in solely HPG (Figure 1).

## DISCUSSION

In postpartum dairy and beef cattle, colonization by microbial infections in the reproductive system can have a negative impact on reproduction. For

instance, uterine infections brought on by harmful bacteria found in dairy cows are common and have an impact on fertility through mediating anovulation and damaging developing oocytes in a way that hinders fertilization or obstructs natural development (14). Fertility disorders like "repeat breeder syndrome" (RBS) can be brought on by postpartum disease that progresses into a chronic subclinical infection (15). According to Bhat et al. (15), uterine bacterial colonization can result in inflammation, mucosal denudation, alterations in secretion, and embryonic mortality. It can also increase the number of services provided per conception, early culling rates, and days open (16). Infection takes place in the uterus of the cow right after calving. The cow is usually able to clear the infection within a few days after calving but if this does not happen then metritis can develop, but also other forms of uterine disease and inflammation such as subclinical endometritis will take place (17).

Reproductive diseases may arise as a result of the presence or absence of particular bacterial populations in the vagina (5,18,19). Because the microorganisms that live there constantly contact with the host mucosa, they serve as the first line of defense against sperm and ascending infections in the bovine reproductive tract. The vaginal microenvironment has recently been suggested to play a significant role in bovine fertility (20-22), despite the fact that research on the bacteria residing in the bovine reproductive tract has historically focused on pathogen populations that colonize the uterus during the postpartum period (12). The abnormalities in the vaginal communities in human females were demonstrated to have been linked to reproductive diseases like pelvic inflammatory illness, miscarriages, and preterm births by Digiulio et al. (23). Therefore, it is crucial to investigate the vaginal microbial ecology in order to comprehend the possible impact of a persistent and disrupted postpartum microbiota on mother health (23), as well as cow health.

Regarding the reproductive performance dairy cows affected by uterine diseases according to the results of existence of microorganisms in the genital canal environment was controversial. In the current study, we associated failed pregnancy outcomes with possible subclinical endometritis based on a rate of 100% bacterial isolation from the vaginal samples of RBG. Contrary to our assumption, Sens and Heusier (24) highlighted positive isolation of microorganisms was not one of the main evidence to subclinical endometritis. Moreover, Paiano et al. (25) demonstrated that 40% of genital channel samples from subclinic endometritis cows did not exhibit bacterial isolation. On the other hand, it was interpreted that the 76% bacterial isolation rate determined in HPG was too high to be underestimated. The positive bacterial microflora of genital canal was thought to support pregnancy in line with Sens and Heusier (24), and Gilbert and Santos (26).

Consistent with our findings, Firmicutes and Proteobacteria were reported the most commonly bacterial phyla found in the vagina of dairy cow by Laguardia-Nascimento et al. (27) and Nesengani et al. (28), but not for Bacteroidetes. The cause of repeat breeder syndrome are categorized into three main reasons comprising ovulation delay, uterine microbiota and luteal deficiency (29). The abundances of bacterial communities that fluctuate during different phases of the estrous cycle in cows are influenced by either high oestrogen or low progesterone levels (30). Research has shown that around days 10–12 after insemination, milk and plasma progesterone concentrations are lower in cows that do not conceive than in animals that conceive over the same period. The consistently low progesterone concentration throughout the estrous cycle is associated with pregnancy failure. Ault et al. (21) reported that the increase of Firmicutes in the vagina was attributed to the decrease in progesterone concentration. However, in this study, it was determined that the abundance of the Firmicutes phylum was equal in both the RBG and the HPG, in line with Moreno et al. (11).

However, the relative abundance of Proteobacteria

(n=32) in HPG comparing with the RBG was associated with an increase in progesterone in line with the declaration of Ault et al. (18). The results suggested that fluctuation of vaginal bacterial population is dependent on the circulating steroid hormones (31). In contrast to the findings of Ault et al (18), the relative abundance of Protobacteria phylum compared with Firmicutes in RBG in the current study was associated with repeat breeder syndrome (RBS) due to being the one of the three phyla not only involved in postpartum uterine infections but also commonly associated with bovine necrotic vulvovaginitis (5, 32). Parallel to Morena et al. (11), Protobacteria (N=21) was determined the most abundant pylum with following Firmicutes (n=14), and Actinobacteria (n=3).

Postpartum disease can evolve into chronic subclinical infection, causing fertility disorders such as the RBS (15). Furthermore, it has been reported that subclinical lesions and inflammation caused by microbiota alteration in the genital system canal can have negative effects during the transportation of spermatozoa, sometimes hindering the formation of fertilization (33). Within first two weeks of calving, up to 40% of animals having a kind of metritis and in 10-15% of these animals infection persists for at least another 3 weeks causing chronic uterine endometritis was a general agreement (1). Moreover, 30-35% of cows have subclinical endometritis between 4 and 9 weeks postpartum (34). Salasel et al. (35) reported that subclinical endometritis was one of the main reason of cows to become repeat breeder. Actinobacteria, Firmicutes and Proteobacteria phyla were reported as an intrauterine bacteriological findings in repeat breeder cows by Pothmann et al. (36). In our study, the same phyla emphasized by Pothmann et al. (36) were determined in both cultivable aerobic vaginal microbiota of RBG and HPG. In the current study, RBG presented numerically lower richness (38 identified species) whereas 48 bacterial species were identified among HPG in line with the Paiano et al.'s (25) results. In terms of the numerically variety of bacteria present in the genital canal, researches indicated that healthy cows possessed a more diverse microbiota in comparison to those suffering from subclinical and chronic endometritis (11, 25).

*Sphingomonas paucimobilis*, *Flavimonas oryzihabitans* dominance in both groups did not make sense regarding reproduction when we evaluated the first two most frequently isolated species for both groups. The most frequently isolated *Bacillus* genus with the rate of 36.84% in RBG were found to be concordent with a study conducted in Brazil which declared the most frequent genus as *Bacillus* in cows suffering from subclinical endometritis (25). However, Ballas et al. (37) demonstrated that *Bacillus* isolation rate from the genital channel samples of healthy cows was more than the cows suffering from endometritis. Whereas *Bacillus* genus with the rate of 31.25% in HPG was too high to ignore. The significant of the presence of *Bacillus macerans* and abundance of *B. brevis* (13.15%) in RBG when compared to HPG in the current study regarding pregnancy should be examined deeply in further studies.

The relative abundance of *Escherichia* genus and *Escherichia coli* with the rates of 7.89% and 7.90% in RBG was found to overlap the study reported the presence of *Escherichia* and *Truperalla* associated with clinical endometritis (38). The presence of numerous adhesion molecules in *E. coli*, particularly its adhesion to the genital region via the fimH gene, formation of biofilm with Type 1 pili, and its exotoxins, have been shown to increase the severity of uterine infections in studies (39). However, in our study, *E. coli* isolation in RBG was not attributed to clinical endometritis because no clinical signs observed in cows. However, the abundance of subclinical uterine colonization of *E. coli* was commented to be considerable due to decreasing the fertility by damaging uterus and inducing ovarian dysfunction by influencing to some degree the pulsatile secretion of luteinizing hormone, the lifespan of corpus luteum and delaying uterine inovulation (11,31).

Pascottini et al. (40) demonstrated that cows with subclinical endometritis had greater

relative abundance of *Corynebacterium* spp., and *Staphylococcus* than cows with clinical endometritis. Although the rate of 7.89% abundance was determined in RBG, and *Corynebacterium pseudodiphtheriticum* was determined in only RBG, the abundance of Corynebacterium genus was thought to be no effect on reproduction. Competible with our comment, Pothmann et al. (36) reported the abundance of Corynebacterium genus with the rate of 20.7% and when the researchers measure progestrone and eastridiol and combined with ultrasonographic asssessment of ovaries, the results indicated an ovarian activity was observed in 95% of the RB cows suffering from subclinical endometritis. In contrast to those, there were studies indicated the association of subclinical endometritis with Corynebacterium genus (41). The abundance of *Stenotrophomonas maltophilia* was not thought to be associated with reproduction problems due to the rate of bacteria in HPG was higher than RBG in the current study.

As a result, we thought that the bacterial diversity of the bovine vagina, the differences in vaginal microbial composition, and the dynamics of vaginal bacterial communities may be associated with reproductive failures and successes. This study identifies differences based on the phylum composition of the naturally occurring aerobic cultivable bacterial communities in the vagina of RB and HP cows. Furthermore, this study also contributes to the knowledge of the species-level local aerobic cultivable bacterial consortium in the vagina of RB and HP cows. The outcomes of the study provides fundamental information that serves as a resource in designing strategies, especially those aimed at enhancing reproductive success.

## ETHICS COMMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## REFERENCES

1. Sheldon IM, Dobson H. Postpartum uterine health in cattle. Anim Reproduct Sci, 2004; 82: 295-306

2. Goncagul G, Intas KS, Kumru IH, Intas DS. Prevalence and accompanying signs of pneumovagina and urovagina in dairy cows in the Southern Marmara region. Tierärztliche Praxis Ausgabe G: Großtiere/ Nutztiere, 2012; 40: 359-366

3. Jeon SJ, Cunha F, Ma X, Martinez N, Vieira-Neto A, Daetz R, et al. Uterine microbiota and immune parameters associated with fever in dairy cows with metritis. PLoS ONE, 2016; 11: e0165740.

4. Lima FS, Oikonomou G, Lima SF, Bicalho ML, Ganda EK, de Oliveira Filho JC, et al. Prepartum and postpartum rumen fluid microbiomes: Characterization and correlation with production traits in dairy cows. Appl Environ Microbiol, 2015; 81: 1327-133.

5. Bicalho M, Machado VS, Higgins CH, Lima FS, Bicalho RC. Genetic and functional analysis of the bovine uterine microbiota. Part I: Metritis versus healthy cows. J Dairy Sci, 2017; 100: 3850-62.

6. Williams EJ, Fischer DP, Pfeiffer DU, England GC, Noakes DE, Dobson H, et al. Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. Theriogenology, 2005; 63: 102-17.

7. Appiah, MO, Wang J, Lu W. Microflora in the reproductive tract of cattle: a review. Agriculture, 2020; 10: 232.

8. Arthur GH, Noakes DE, Pearson H, Perkinson TJ. Veterinary Reproduction and Obstetrics. Theriogenology (4th Ed.), Bailliere Tindal Great Britain, UK, 1996; 291-301.

9. Lobago F, Bekana M, Gustafsson H, Kindahl H. Reproductive performances of dairy cows in smallholder production system in Selalle, Central Ethiopia. Trop Anim Health Prod, 2006; 38: 333-42.

10. Wang J, Sun C, Liu C, Yang Y, Lu W. Comparison of vaginal microbial community structure in healthy and endometritis dairy cows by PCR-DGGE and real-time PCR. Anaerobe, 2016; 38: 1-6.

11. Moreno CG, Luque AT, Galvão KN, Otero MC. Bacterial communities from vagina of dairy healthy heifers and cows with impaired reproductive performance. Research in Vet Sci, 2022; 142: 15-23.

12. Bicalho M, Santin T, Rodrigues M, Marques C, Lima S, Bicalho R. Dynamics of the microbiota found in the vaginas of dairy cows during the transition period: Associations with uterine diseases and reproductive outcome. J Dairy Sci, 2017; 100: 3043-58.

13. Katagiri S. A new approach to repeat breeding in cows: treatments targeting the endometrial growth factor-cytokine network. Thai J Vet Med Suppl, 2011; 41: 51-3.

14. Gilbert RO. Symposium review: Mechanisms of disruption of fertility by infectious diseases of the reproductive tract. J Dairy Sci, 2019; 102: 3754-65.

15. Bhat FA, Bhattacharyya HK, Hussain SA. White side test: A simple and rapid test for evaluation of nonspecific bacterial genital infections of repeat breeding cattle. Vet Res Forum, 2014; 5: 177.

16. Mahnani A, Sadeghi-Sefidmazgi A, Cabrera VE. Consequences and economics of metritis in Iranian Holstein dairy farms. J. Dairy Sci, 2015; 98: 6048-57.

17. Bittar JH, Pinedo PJ, Risco CA, Santos JE, Thatcher WW, Hencken KE, et al. Inducing ovulation early postpartum influences uterine health and fertility in dairy cows. J Dairy Sci, 2014; 97: 3558-69.

18. Ault TB, Clemmons BA, Reese ST, Dantas FG, Franco GA, Smith TPL, et al. Bacterial taxonomic composition of the postpartum cow uterus and vagina prior to artificial insemination. J Anim Sci, 2019; 97: 4305-13.

19. Galvao KN, de Oliveira EB, Cunha F, Daetz R, Jones K, Ma Z, et al. Effect of chitosan microparticles on the uterine microbiome of dairy cows with metritis. Appl Environ Microbiol, 2020; 86: e01066-20.

20. Clemmons BA, Reese ST, Dantas FG, Franco GA, Smith TPL, Adeyosoye OI, et al. Vaginal and uterine bacterial communities in postpartum lactating cows. Front Microbiol, 2017; 8: 1047.

21. Ault TB, Clemmons BA, Reese ST, Dantas FG, Franco GA, Smith TPL, et al. Uterine and vaginal bacterial community diversity prior to artificial insemination between pregnant and nonpregnant postpartum cows. ASOS, 2019; 97: 4298-304.

22. Messman RD, Contreras-Correa ZE, Paz HA, Perry G, Lemley CO. Vaginal bacterial community composition and concentrations of estradiol at the time of artificial insemination in Brangus heifers. J Anim Sci, 2020; 98: 178.

23. Digiulio DB, Callahan BJ, Mcmurdie PJ, Costello EK, Lyell DJ, Robaczewska A, et al. Temporal and spatial variation of the human microbiota during pregnancy. Proc Natl Acad Sci USA, 2015;112: 11060-5.

24. Sens A, Heuwieser W. Presence of Escherichia coli, Trueperella pyogenes, ahemolytic streptococci, and coagulase-negative staphylococci and prevalence of subclinical endometritis. J Dairy Sci, 2013; 96: 6347e54.

25. Paiano RB, Moreno LZ, Gomes VTDM, Parra BM, Barbosa MR, Sato MIZ, et al. Assessment of the main pathogens associated with clinical and subclinical endometritis in cows by culture and MALDI-TOF mass spectrometry identification. J Dairy Sci, 2022; 105: 3367-76.

26. Gilbert RO, Santos NR. Dynamics of postpartum endometrial cytology and bacteriology and their relationship to fertility in dairy cows. Theriogenol, 2016: 85: 1367-74.

27. Laguardia-Nascimento M, Branco KMGR, Gasparini MR, Giannattasio-Ferraz S, Leite LR, Araujo FMG, et al. Vaginal microbiome characterization of Nellore cattle using metagenomic analysis. PLoS One, 2015; 10: e0143294.

28. Nesengani LT, Wang J, Yang Y, Yang L, Lu W. Unravelling vaginal microbial genetic diversity and abundance between Holstein and Fleckvieh cattle. RSC Advances, 2017; 7: 56137-43.

29. Pérez-Marín CC, Quintela LA. Current insights in the repeat breeder cow syndrome. Animals, 2023; 13: 2187.

30. Otero C, Silva De Ruiz C, Ibañez R, Wilde OR, De Ruiz Holgado AAP, Nader-Macias ME. Lactobacilli and enterococci isolated from the bovine vagina during the estrous cycle. Anaerobe, 1999; 5: 305-7.

31. Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biolof Reprod, 2009; 81: 1025-32.

32. Elad D, Friedgut O, Alpert N, Stram Y, Lahav D, Tiomkin D, et al. Bovine necrotic vulvovaginitis associated with Porphyromonas levii. Emerg Infect Dis, 2004; 10: 505-7.

33. El-Khadmwy HH, Ahmed WM, Hanafi M. Observations on repeat breeding in farm animals with emphasis on its control. J Reprod Infertil, 2011; 2: 1-7.

34. LeBlanc SJ. Postpartum uterine disease and dairy herd reproductive performance: a review. Vet J, 2008; 176: 102-14.

35. Salasel B, Mokhtari A, Taktaz T. Prevalence, risk factors for and impact of subclinical endometritis in repeat breeder dairy cows. Theriogenology, 2010; 74: 1271-8.

36. Pothmann H, Prunner I, Wagener K, Jaureguiberry M, de la Sota RL, Erber R, et al. The prevalence of subclinical endometritis and intrauterine infections in repeat breeder cows. Theriogenology, 2015; 83: 1249-53.

37. Ballas P, Reinländer U, Schlegl R, Ehling-Schulz M, Drillich M, Wagener K. Characterization of intrauterine cultivable aerobic microbiota at the time of insemination in dairy cows with and without mild endometritis. Theriogenology, 2021; 159: 28-34.

38. Poole RK, Soffa DR, McAnally BE, Smith MS, Hickman-Brown KJ, Stockland EL. Reproductive microbiomes in domestic livestock: Insights utilizing 16S rRNA gene amplicon community sequencing. Animals, 2023; 13: 485.

39. Sheldon IM. Genes and environmental factors that influence disease resistance to microbes in the female reproductive tract of dairy cattle. Reprod Fertil Develop, 2015; 27: 72-81.

40. Pascottini OB, Van Schyndel SJ, Spricigo JW, Rousseau J, Weese JS, LeBlanc SJ. Dynamics of uterine microbiota in postpartum dairy cows with clinical or subclinical endometritis. Scientific reports, 2020; 10: 12353.

41. Miranda-CasoLuengo R, Lu J, Williams EJ, Miranda-CasoLuengo AA, Carrington SD, Evans AC, et al. Delayed differentiation of vaginal and uterine microbiomes in dairy cows developing postpartum endometritis. PloS one, 2019; 14: e0200974.