Dynamics of Vaginal Microbiota During Estrous Cycle in Cows through Metagenomic Approach

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Research

Keywords: Vaginal Microbiota, Estrous Cycle, Cows, Metagenomic

Posted Date: May 20th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-508883/v1>

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Abstract

Background: Using 16s rRNA sequencing of the V3-V4 hypervariable region, the present study is aimed to check vaginal microbiota diversity throughout different stages of the estrous cycle, with attention to hormonal changes and microorganism diversity. Metagenomic research was conducted on vaginal swab samples obtained from healthy cows' at different stages of the estrous cycle.

Results: Total sixteen cows were synchronized with double PG regime. Nine cows demonstrated estrus within 96 hours and were eligible for the experiment. Vaginal samples for metagenomics and blood samples for hormonal analysis were collected during estrus, metestrus, diestrus and proestrus of synchronized Estrous cycle. The study's findings revealed that the diestrus phase has a different diversity than the other three estrous cycle phases, implying that hormones affect bacterial diversity. Proteobacteria and Firmicutes are the most abundant phyla at the phylum level, accounting for 94 % of bacterial diversity. Actinobacteriota, Patescibacteria, Cyanobacteria, Bacteroidota, and others are fewer common phyla. Proteobacteria are most common throughout the estrus, metestrus, and proestrus stages of the estrous cycle at the phylum level, there was no discernible distinction between the follicular and luteal phases. After statistical correction, Bacillaceae, Alcaligenes, and Enterobacteriaceae & Morganellaceae families are more significant. At the diestrus stage, the Family Enterobacteriaceae is lower than at other stages; otherwise, all statistically significant genera are high at diestrus stages. The luteal phase had higher levels of Micrococcus, Stenotrophomonas, UGC-010, Massilia, and Methylobacillus than the follicular phase, however, statistical analysis revealed no substantial difference between the two phases. Lactobacillus genus is present on two samples including the estrus stage and diestrus stages.

Conclusions: This study represents an important step towards the understanding of microbial diversity within different stages of the estrous cycle of the dairy cow. The study results revealed dynamics of microbiota during estrous cycle.

Background

The bovine vaginal ecosystem of a cow (*Bos taurus*) harbors a dynamic mixture of aerobic facultative anaerobic and strict anaerobic micro-organisms [1, 2]. Identifying the composition of vaginal microbiota can provide an important understanding of the reproductive health of bovines. This can also pave a way for effective therapeutic interventions for bovine health improvement [3].

There are reports of the bovine reproductive tract where microflora populations differ between luteal and follicular stages [4, 5, 6]. For instance, in the comparison of microbial flora during the follicular and luteal phase, the vaginal microflora of cattle was dominated by *E. coli*, *Aerococcus vaginalis*, *Aerococcus viridans*, *Haemophilus Somnus*, *Streptococcus pluranimalium*, *Sphingomonas roseiflava*, *Psychrobacter marincola*, and *Lactobacillus spp*, as revealed in the laboratory using PCR-DGGE [2, 4]. A recent study by Quereda et al [7] remarked the amplest microbiota phyla detected in dairy heifer's vaginal microbiota during the luteal and follicular phase of the estrous cycle where Tenericutes, Firmicutes, and

Bacteroidetes, showed 75% relative abundance. However, genus *Lactobacillus* existed at an occasional relative abundance throughout the estrous cycle. Ault et al. [8] have also observed variations in *Fusobacterium*, *Tenericutes*, and *Verrucomicrobia* deviation between pregnant and non-pregnant cows. Further, they represented as because the reproductive tract prepares for gestation, a significant decrease in diversity of the microbiota throughout the luteal phase.

The distribution of microflora within a bovine's uterus can have a significant effect on cattle health. Studying this microflora residing in the different phases of the estrous cycle will enable us to view plausible microbiota which could be potential candidates for probiotics, and eventual application to new therapeutics to treat infection throughout reproductive protocol by understanding microbial diversity [7]. Additionally, restoring the ecological balance may decrease the proliferation of pathogens within the bovine's vagina [9]. Understanding normal microbiota composition during various phases of estrous cycle can further help in restoring normal vaginal balance in case of various infections like metritis, pyometra, and endometritis etc., [10]. With the help of metagenomics [11], it has now become very easy to understand the diversity difference in various phases of Estrous cycle for harnessing the power of beneficial vaginal probiotic consortium [12].

India has distinct breeds of cattles, and studies on Gujarat breeds of cow specifically Gir cow and Holstein Firestein crosses, has not been reported yet, Gir cow usually habitat within the Saurashtra region of Gujarat, its acknowledged for the tolerance to stress conditions and resistance to varied tropical diseases. Gir cow has the capacity of yielding more milk with less feeding. The studies on vaginal microbiota of normal estrous cycle are not explored for potential probiotic microbes for health applications. Therefore, the objective of the present study was to investigate the taxonomic microbial diversity during the different stages of the estrous cycle which include the phases of Proestrus, Estrus, Diestrus, and Metestrus with the use of 16s rRNA amplicon metagenome sequencing of the V3-V4 hypervariable region.

Methods

a. Sample Collection from Animals

Two commercial dairy farms nearby with a minimum of 50 animals were enrolled for the study. At both, farms animals were fed *ad libitum* with wheat hay, lucerne, and green grass. The concentrate mixture (cottonseed, groundnut, soybean, maize, etc) was fed at 2 kg per animal after boiling. Animals in both the farms were screened for brucella, TB, and JD before starting the experiment and were found negative. Sixteen multiparous cows with median 3 (1 to 5) lactations and 80 (70 to 95) days in milk were selected initially for the study. All cows were evaluated for reproductive health with rectal palpation and cervicovaginal discharge. Cows were administered 500 mcg cloprostenol (Pregma, Intas pharma, India) intramuscularly at 10-day intervals to promote lysis of corpus luteum and estrus synchronization. Using behavior signs, cervico-vaginal estrus discharge, rectal palpation, and synchronized estrus within

96 hours after second cloprostenol injection was considered inclusion criteria for final enrollment of cows. Out of 16, nine cows fulfilled the above criteria and included in the study.

Vaginal Samples

Four vaginal samples per cow were collected during the estrus (day 0: D0), metestrus (day 04: D4), diestrus (day 12: D12), and proestrus (day 16: D16) phase of estrous cycle. Before taking the vaginal samples, back-raking was performed followed by external genitalia were cleaned with 4% chlorhexidine and dried with tissue paper. Sterile vaginal Swab (17214/2950 Swab, Eppendorf Pvt Ltd., India) were used to collect the vaginal samples from the fornix of the vagina. The samples were collected rotating swab towards clock viz direction for the 30s during four phases of the estrous cycle in cows. The samples were transported immediately in an icebox at 4 °C at the laboratory. Before processing, the samples were labeled according to the stage of the estrous cycle, cow number and farm identification.

Blood Samples

Collection of blood samples on the respective days of the harmonized estrous cycle were day D0, D4, D12, and D16 from the jugular vein using 8 ml EDTA vacutainers. Blood was transported at 4°C to the laboratory and plasma extracted after centrifuging at 4000 rpm (Thermo Scientific Sorvall X4R Pro-MD, India) for 5 minutes and separated plasma samples were labeled and stored in 2ml plasma storage vials at -20°C until analysed for progesterone (P4) concentration by chemiluminisence immune assay (CLIA) method in a commercial laboratory (Gaievski et al 2018). Plasma P4 concentration < 1.0 ng/ml defined estrus and P4 concentration above > 1.0 ng/L defined other than estrus phase. The intra and inter assay coefficient of variation for P4 was 8.1 % and 8.6 %, respectively.

Nucleic acid Extraction and Amplification:

Vaginal swab samples from the nine cows were collected during the Estrous cycle at four different points (D0, D4, D12, and D16) for DNA isolation. DNA isolation from the swab sample was done using Qiagen Stool DNA isolation kit (cat. No. 51504) using the protocol provided in the manual of the kit. DNA quantification and purity were checked using the QIAxpert System. DNA was run on 0.8% agarose gel electrophoresis to check DNA integrity.

DNA was amplified using a specific primer of V3-V4 region of bacterial 16s rRNA gene Barcoded Fusion Forward Primer and Reverse Primer in Table 3 using thermal cycle condition. Denaturation: 1 min at 95 °C, Annealing: 30 Sec at 58°C, Extension: 45 secs at 75 °C (30cycles). PCR product was run on 2% agarose gel electrophoresis to confirm the product size.

Library Preparation and Sequencing:

From the amplified product some of the products of the 16s rRNA gene showed nonspecific size amplification. Size-specific product (~450bp) purification was done using the E-Gel CloneWell II agarose gels. E-Gel CloneWell II agarose gel contains two comb systems. The sample was loaded into the upper

well and electrophorese until the desired band came into the lower well. Then just simply the band is pipetted out and quantified using Qubit dsDNA HS Assay Kit with Qubit 2.0 Fluorometer. Further, remaining PCR products are purified using AMPure XP beads with a 0.9X ratio according to the user guide. After purification, Quantification was done using Qubit dsDNA HS Assay Kit with Qubit 4.0 Fluorometer before pooling of library and then sequencing was done using ion 5s platform using 530chip, with 400bp chemistry.

Amplicon Sequence analysis:

Raw reads were processed using a Perl script followed by prinseq lite. Data was filtered taking the quality value 25 and trimming of smaller sequences lower than 200bp. After filtration, the data were analysed using the software QIIME 2-2020.8 [13] with default parameters until stated otherwise. DADA2 was used for the denoising and demultiplexing of the reads. The taxonomical analysis was done using the SILVA database [14]. OTU clustering was done using 99% identity. Jaccard, Bray-Curtis, unweighted and weighted Unifrac distance were calculated to observe the difference in the community.

Statistical Analysis

Alpha Rarefaction curve was generated using different metrics Shannon, observed feature, and Faith_Pd. Alpha diversity analysis was done using Kruskal-Wallis (pairwise) statistics. Beta diversity between the groups was calculated using permanova pairwise statistical analysis. % Relative abundance was calculated from taxa generated from the QIIME 2.0. The Taxonomic data were further analysed using the STAMP software [15] and statistical analysis was carried out using Multiple test ANOVA with log transform. The statistical correction was done using the Benjamini-Hochberg FDR test [16]. Data were analysed using a Confidence interval of 95%. Effect of farm and breed of cows (Gir vs HF cross) were non-significant and removed from further analysis ($P < 0.05$).

Results

Diversity Analysis

The Rarefaction curve proclaimed that the achieved sampling size and sequencing depth were adequate to observe the complete diversity of the microbiota. Alpha rarefaction curve reached a plateau for all the samples at the sequencing depth of 5000 reads. Figure 1. The D16 stage of the Estrous cycle has shown more alpha diversity compared to the D0, D4, and D12 stages ($P < 0.05$), where the trend can be observed by the alpha diversity boxplot in Figure 2. Alpha diversity of the D16 stage has shown a significant difference from the D4 stage ($P = 0.007$) Proestrus stage also differed significantly from the D0 stage. ($P = 0.043308$; Table 1) The diversity between the Follicular and Luteal stage also shows a significant difference. ($P = 0.031341$).

Beta diversity between four stages of the estrous cycle, cattle breeds, and farms was calculated using pseudo-F PERMANOVA pairwise statistical method. Alpha Diversity and Beta diversity of estrous cycle of

cows of two farms were nonsignificant ($P > 0.05$) hence the further analysis was carried out using a cow as a unit of analysis. Beta diversity of D12 and D16 stages differed significantly from D4 ($p < 0.01$ and 0.023 , respectively). Considering two phases i.e., follicular and luteal phase; of four estrous stages shown no significant difference.

Taxonomical analysis

Data of taxonomic analysis was represented as % mean abundance. Cow vaginal bacteria were found to belong to the two most abundant phyla Proteobacteria, and Firmicutes (Figure 3) which comprise 94% of bacterial diversity. Other less abundant phyla are Actinobacteriota, Patescibacteria, Cyanobacteria, Bacteroidota, Verrucomicrobiota and Desulfobacterota (Figure 3). Only Bacteroidota, Firmicutes, Proteobacteria, and Verrucomicrobiota showed significant differences across the estrous cycle stages (Figure 4). The Diestrus stage has a 1.2% mean proportion of Bacteroidota and a 40% mean proportion of Firmicutes which is higher than the other estrous stages. Proteobacteria is present across all the stages but the D12 stage has a lower mean proportion compared to other estrous stages (Figure 4). At the phylum level, the D12 diestrus stage shows significantly different diversity compared to other stages. But when the Follicular (D 16 and D0) and Luteal (D 04 and D 12) phases were compared there was no significant difference between phyla ($P = 0.025$). At the Genus level analysis showed that the D 12 stage showed a greater level of *Bacillus* (13% Mean population) compared to other groups (Figure 5). Estrus (D0) stage also showed greater *Bacillus* compared to that of the other two stages D4 and D16. At D 12 *Alcaligenes* (1.3% Mean abundance) is also greater than D16 and D4 stages. Metestrus (D4) stage has the highest % mean abundance (83%) of the Enterobacteriaceae family followed by D16 and D0. The Enterobacteriaceae family has the lowest % mean abundance at the D12 Stage (Figure 6). If we divide the estrous stage into Follicular and Luteal phase. *Micrococcus*, *Stenotrophomonas*, UGC-010, *Massilia*, *Methylobacillus* genus was observed statistically significant at luteal phase and *Pseudomonas* genus in the Follicular phase (Figure 7).

Discussion

The present study demonstrated microbial diversity of Holstein-Friesian and Gir cow during the estrous cycle under Indian conditions. Cows from two farms were included in this report, although there was no difference in taxonomic analysis between the two farms. This may be due to close proximity of farms and optimal feeding and management practices. There are reports [7] on cow microbiota during the estrous cycle, however, are from herds of North America or Europe. In our research, we looked at data from four different phases (D0, D4, D12, and D16), which are divided into two groups: follicular (D16 and D0) and luteal (D4 and D12). The microbiological diversity across the stages may be influenced by different hormone concentrations present during the estrous cycle phases [6]. Similarly, our research also found that the four estrous stages have an effect on microbial diversity. Quereda et al [6] have demonstrated that the vaginal microbiota of cows was statistically different at the follicular and luteal phases of the estrous cycle in dairy heifers.

In this analysis, there is a substantial difference in alpha and beta diversity between the Luteal and Follicular phases. No significant phyla were observed, however, *Micrococcus*, *Stenotrophomonas*, UGC-010, *Massilia*, *Methylobacillus*, and *Pseudomonas* genus was observed statistically significant in the Luteal and follicular phase. There was no substantial genus after statistical correction of the results. Only the diestrus stage of the luteal process reveals a statistically crucial distinction in the microbiota abundance. Lactic acid bacteria (LAB) are known to persist in human vaginal microbiota and inhibit opportunistic pathogen proliferation [17, 18]. Although in other mammals, LAB does not dominate as vaginal microbiota [19,20]. Though shift of principal microorganisms occur during the bovine's ovarian cycle [21]. However, the most mentioned genera of bovine's vagina LAB are *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella*, which are phylogenetically close to each other [22] and usually measured together as the *Lactobacillus* group [23].

Women's vaginal microbiota mainly consist of *Lactobacillus* genus [18, 24], however, bovine shows a low abundance of *Lactobacillus* genus [6], but high diversity of the Proteobacteria and Firmicutes. Quereda et al [6] observed that *Lactobacillus* is not dominant in the microflora of cow heifers which supports the results of our study. Swartz et al [24] presented that the most profuse genera were *Streptobacillus* spp. and *Aggregatibacter* spp. *Lactobacillus* spp. were detected in 90% of cows and 80% of ewe samples. In our study, there are four phyla Bacteroidota, Firmicutes, Proteobacteria, and Verrucomicrobiota which are statistically significant after statistical correction by the Benjamini-Hochberg FDR test. Firmicutes and Proteobacteria consist of 94% of the phyla of the vaginal diversity during the estrous cycle. Swartz et al [24] presented that Ewes and cows were predominantly colonized by the Proteobacteria, Bacteroidetes, and Fusobacteria. So many reports are supporting the results of our study. Giannattasio-Ferraz et al [25] they also demonstrated that most superabundant phyla are firmicutes that contain 40-50% of the diversity followed by Proteobacteria, Bacteroides, and Actinobacteria in vaginal microflora. Differences in bacterial diversity were observed between these studies because of Breed, Feed, environmental condition, geological location, age, etc. (A common vaginal microbiota composition among breeds of *Bos taurus indicus* (Gyr and Nellore). Nellore Cattle phyla consist of Firmicutes (~40–50%), Bacteroidetes (~15–25%), and Proteobacteria (~5–25%). (Vaginal Microbiome Characterization of Nellore Cattle Using Metagenomic Analysis). In the study of Ault and Colleagues observed that verrucomicrobiota has high % abundance in postpartum cows, which became pregnant after insemination compared to cow which did not get pregnant. {Vaginal microbiota changes during the estrous cycle in Dairy Heifers} observed that there are 4 families and 17 genera showed relative abundance >1%. f_Leptotrichiaceae, f_Corynebacteriaceae, Ruminococcaceae UCG-005, Mycoplasma, Helcococcus, Bacteroides, Campylobacter, Porphyromonas, Histophilus, Ureaplasma, Rikenellaceae RC9 gut group, f_Lachnospiraceae, Streptococcus, Alistipes, Facklamia and coprostanoligenes group were the most abundant families and genera observed. In our study, we observed only two significant genera and two significant families. The only significant genera and family were *Bacillus*, *Alcaligenes*, f_Enterobacteriaceae, and f_Morganellaceae. *Micrococcus*, *Stenotrophomonas*, UGC-010, *Massilia*, *Methylobacillus* genus was observed statistically significant at luteal phase and *Pseudomonas* genus in

the Follicular phase but after statistical correction of data there was no significant genus was observed between follicular and luteal phase.

Conclusions

The most abundant bacterial phyla in the vaginal microbiota of cows were shown to be Proteobacteria and firmicutes which comprises of 94% relative abundance of bacteria. Microbial community composition was found to be highly variable between across the estrous cycle of dairy heifers. Lactobacillus was not one of the most common genera found.

Identification of bacterial communities during different stages of the estrous cycle can lead to the creation of new approaches such as probiotic treatment and the introduction of microbial strains that have a positive impact on fertility success. The heterogeneity of group composition between individuals was verified in this study, suggesting the need for larger experimental sizes in future research.

Abbreviations

TB: Tuberculosis; JD: Johnes Disease; D0: estrus; D4 Diestrus; D12: Metestrus; D16: Proestrus

Declarations

Ethics declarations

All experimental designs and protocols were approved by the Animal Ethics Committee of the Kamdhenu University and as per the guidelines for animal research (No. KU/DR/IAUC/0724).

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

Project name: Quantitative Insights into Microbial Ecology (QIIME)

Project home page: e.g. <https://qiime2.org/>

Archived version: QIIME 2.2020

Operating system: Linux

Programming language: C language

License: © Copyright 2015–, QIIME development team.

The datasets used and/or analysed during the current study are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by the Gujarat State Biotechnology Mission, Department of Science and Technology, Government of Gujarat, India (Grant No. 2018YFD0500703, 2017YFD0701604).

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Author's contributions

The experimental scheme was designed by VS, MJ, and CJ. VS, KP, and KP participated in the experiment process and assisted in sampling. The analysis of experimental data and the making of charts were completed by KP, Nitin and AP completed the initial draft, PG rewrote draft. VS, and MJ completed the overall modification of the manuscript. CJ and DP improved and polished the language of the article. DP, MJ, and CJ provided the necessary experimental equipment and key guidance during the experiment process. The authors read and approved the final manuscript.

Acknowledgements

We greatly acknowledge Gujarat State Biotechnology Mission, Department of Science and Technology, Gandhinagar, Gujarat, India for providing financial support to carry out this research work. We are also thankful to the owners of the Dairy farms who permitted us to get samples from the cows.

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Table

Table 1: Difference between Alpha diversity of four stages of estrous cycle using Kruskal-Wallis (pairwise) statistics using faith phylogenetic diversity group significance.

Group 1	Group 2	H	p-value	q-value
Diestrus (n=7)	Estrus (n=9)	2.04216807	0.153006666	0.183608
Diestrus (n=7)	Metestrus (n=9)	2.355742297	0.124822457	0.183608
Diestrus (n=7)	Proestrus (n=8)	7.084821429	0.007773957	0.046643739
Estrus (n=9)	Metestrus (n=9)	0.001949318	0.964783978	0.964783978
Estrus (n=9)	Proestrus (n=8)	4.083333333	0.043308143	0.108583657
Metestrus (n=9)	Proestrus (n=8)	3.703703704	0.054291828	0.108583657

Figures

Figure 1

Alpha Rarefaction Curve of all the samples at the depth of 5000

Figure 2

Alpha Diversity box plot of all the stages at sequence depth of 5000

Figure 3

Beta Diversity of four stages of Estrous cycle (D12: Diestrus; D0: Estrus; D04: Metestrus and D16: Proestrus) at Phylum Level in cows

Figure 4

Significant phyla after statistical analysis using multiple test ANOVA and statistical correction done using Benjamini-Hochberg FDR of data Beta Diversity of between four stages of estrous cycle of cows (n = 09) at Phylum Level

Figure 6

Significant Genus after statistical analysis using Multiple test ANOVA and statistical correction was done using Benjamini-Hochberg FDR of data Beta Diversity of four stages of estrous cycle of cows (n =09) at Genus Level

Figure 7

Significant Genus after statistical analysis using Multiple test ANOVA without statistical correction between follicle and luteal phase of the estrous cycle of cows (n =09).

Figure 8

Principal component analysis (PCA) plot analysis for four stages of estrous cycle in cows (n =09)