Metagenomic Analysis of Bacterial Communities During Estrous Cycle in *Bos Indicus*

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Abstract

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 in *Bos indicus*, with attention to progesterone hormone changes and microorganism diversity. Metagenomic research was conducted on vaginal swab samples obtained from nine healthy Indian Gir cows' at estrus (Day0), metestrus (Day04) and diestrus (Day12) and proestrus (Day16), phases of the estrous cycle. The study's findings revealed that the diestrus phase has a different diversity than the other three estrous cycle phases, implying that progesterone 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 including others are fewer common phyla. After statistical correction, *Bacillaceae, Alcaligenes*, and *Enterobacteriaceae* & *Morganellacea* families are more significant. At the diestrus phase, the Family Enterobacteriaceae is lower than at other three phases; otherwise, all statistically significant genera are high at diestrus phase. The luteal stage had higher levels of *Micrococcus, Stenotrophomonas*, UGC-010, *Massilia*, and *Methylobacillus* than the follicular stages, statistical analysis revealed there is substantial difference between the luteal and follicular stages. Lactobacillus genus is present on two phases including the estrus phase and diestrus phase. This study represents an important step towards the understanding of microbial diversity within different stages of the estrous cycle of the Indian cows.

Introduction

The bovine vaginal ecosystem of a cows (*Bos taurus*) harbors a dynamic mixture of aerobic facultative anaerobic and strict anaerobic micro-organisms (Otero et al. 2000, Santos and Bicalho 2012). Identifying the composition of vaginal microbiota can provide an important understanding of the reproductive health of cows. This can also pave a way for effective therapeutic interventions of cow health improvement (Baker et al. 2018).

There are reports of the bovine reproductive tract where microflora populations differ between proestrus, estrus, metestrus and diestrus stages (Mahesh et al. 2020, Mahesh et al. 2021) or during follicular and luteal phases (Ault et al. 2019, Quereda et al. 2020). For instance, in the comparison of microbial flora during the follicular and luteal phases, the vaginal microflora of cattle was dominated by *Escherichia coli, Aerococcus vaginalis, Aerococcus viridans, Haemophilus somnus, Streptococcus pluranimalium, Sphingomonas roseiflava, Psychrobacter marincola*, and *Lactobacillus spp*, as revealed in the laboratory using PCR-DGGE (Santos and Bicalho 2012, Wang et al. 2018). A recent study by Quereda et al., (2020) remarked the amplest microbiota phyla detected in dairy heifer's vaginal microbiota during the luteal and follicular stage of the estrous cycle where *Tenericutes, Firmicutes*, and *Bacteroidetes*, showed 75% relative abundance. However, genus Lactobacillus existed at an occasional with relative abundance throughout the estrous cycle. Ault et al. (2019) 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 stage.

The distribution of microflora within a dairy cow's uterus can have a relationship with fertility and production. Studying this microflora residing in the different phases of the estrous cycle will enable to view plausible microbiota which could be potential candidates for probiotics, and eventual application to new therapeutics to treat infection throughout reproductive protocols by understanding microbial diversity (Quereda et al. 2020). Additionally, restoring the ecological balance may decrease the proliferation of pathogens within the cow's vagina (Otero, et al. 2000). Understanding these 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., (Moreno et al. 2016). With the help of metagenomics (Alves et al. 2018), 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 (Machado et al. 2012).

India has distinct breeds of cattle, and studies on Gujarat breeds of cows specifically Gir, has not reported yet, Gir cows usually habitat within the Saurashtra region of Gujarat, it's acknowledged for the tolerance to stress conditions and resistance to varied tropical diseases. Gir cows 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 and other health applications. Therefore, the objective of the present study was to investigate the vaginal taxonomic microbial diversity during the different phases of the estrous cycle which include Proestrus, Estrus, Metestrus, and Diestrus with the use of 16s rRNA amplicon metagenome sequencing usingV3-V4 hypervariable region in *Bos indicus*.

Material And Methods

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 animals. Animals in both farms were screened for Brucella, TB, and JD before start of the experiment and were found negative and body condition score between 3 to 3.5. Sixteen multiparous cows (n = 07 from farm 1 and n = 09 from farm 2) with median 3 (ranges from1 to 5days) lactations and 80 (Ranges from 70 to 95 days) days in milk were selected initially for the study. All cows were evaluated for reproductive health with rectal palpation and cervico-vaginal discharge. Cows selected for study were administered 500 mcg cloprostenol (Pregma, Intas pharma pvt. Ltd., India) intramuscularly at 10-day intervals to promote lysis of corpus luteum and estrus synchronization. Using behavioural signs, cervico-vaginal estrus discharge, rectal palpation, and synchronized estrus within 96 hours after second cloprostenol injection were considered inclusion criteria for final enrolment of cows. Out of sixteen cows, nine cows were fulfilled the above criteria and selected for 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 cleaning of external genitalia with 4% chlorhexidine and dried with tissue paper. A sterile vaginal swab (17214/2950 Swab, Eppendorf Pvt ltd., India) were used to collect the vaginal samples from the fornix of the vagina. Samples were taken by spinning a swab in a clockwise manner for 30 seconds. The samples were transported immediately in an icebox at 4 ℃ at the laboratory. Before processing, the samples were labelled according to the phases of the estrous cycle, cow number and farm identification.

Blood collection and progesterone determination

Blood samples were collected on the corresponding days of the synchronizined estrous cycle were day of D0, D4, D12, and D16 from the jugular vein using 8 ml EDTA vacutainers. Blood was transported at 4℃ to the laboratory and plasma extracted after centrifuging at 4000 rpm (Thermo Scientific Sorvall X4R Pro-MD, India) for 5 minutes. Plasma was separated and stored in 2ml storage vials at -20˚C for progesterone (P4) concentration determined by chemiluminisence immune assay (CLIA) method in a commercially available laboratory. Progesterone concentration < 1 ng/ml defined estrus with confirmation of uterine tonicity on rectal palpation, observing vaginal mucus discharge and behavioural signs and progesterone concentration above > 1.0 ng/L with days from synchronized estrus defined other than estrus phase i.e., day 4, day 12 and day 16. The intra and inter assay coefficient of variation for progesterone was 8.1 % and 8.6 %, respectively.

Nucleic acid extraction and amplification

DNA was extracted from vaginal swab samples of the nine cows were collected during the Estrous cycle at four different phases (D0, D4, D12, and D16 DNA isolation was performed using the Qiagen Stool DNA Isolation Kit (cat. no. 51504) and the methodology was performed as outlined in the kit's manual. The QIAxpert System was used to verify DNA quantity and purity. To ensure the integrity of the DNA, it was run on a 0.8 percent agarose gel electrophoresis.

DNA samples were amplified using a specific primer of V3-V4 region specific bacterial 16s rRNA gene Barcoded Fusion Forward Primer and Reverse Primers (Table. S1) using PCR thermal cycle conditions. The PCR reaction included initial denaturation for 3 min at 95°C, followed by 30 cycles at 95°C for 30s, 55° C for 30s and 72°C for 30s, and a final elongation step at 72°C for 5 min. To check the size of the PCR result, DNA samples were run on a 2% agarose gel electrophoresis.

Library preparation and sequencing

Some of the 16s rRNA gene products showed nonspecific size amplification from the amplified product. Size-specific product (~450bp) purification was done using the E-Gel CloneWell II agarose gels, where it has two comb systems. The samples were loaded into the upper well and electrophorese until the desired

band came into the lower well. After that band was 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 (Beckman Coulter, Switzerland) 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 followed by 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 selecting the quality value 25 and trimming of smaller sequences > 200bp. After filtration, the data was analysed using the software QIIME 2-2020.8 (Bolyen et al. 2019) with default parameters until stated otherwise. DADA2 pipeline were used for the denoising and demultiplexing of the reads. The taxonomical analysis was done using the SILVA database (Quast et al. 2013). Operational taxonomic unit (OUT) clustering was done using 99% identity. Jaccrad, Bary Cutris, unweighted and weighted Unifrac distance were calculated to observe the difference in the community.

Statistical analysis

Alpha Rarefaction curve was generated using different metrics observed features, and Faith_Pd. Alpha diversity analysis was performed using Kruskal-Wallis (pairwise) statistics. Beta diversity between the four estrous cycle's phases 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 bioinformatics tool (Parks et al. 2014) and statistical analysis was carried out using multiple test ANOVA with log tra using STAMP V.2.1.3. The statistical correction was performed using the Benjamini-Hochberg FDR test (Benjamini and Hochberg 1995). Data were analysed using a confidence interval of 95%. Effect of farm were non-significant because of having $P<0.05$ and removed from further analysis.

Results

Diversity analysis

The rarefaction curve proclaimed that, graph has achieved sampling size and sequencing depth were adequate to observe the complete diversity of the microbiota (Willis 2019). Alpha rarefaction curve reached a plateau for all the samples at the sequencing depth of 5000 reads. (Fig. 1). The D12 phase of the estrous cycle has demonstrated more alpha diversity compared to the D0, D4, and D16 phases ($P < 0.05$), where the shifts can be observed by the alpha diversity boxplot in Fig. 2. Alpha diversity of the D12 phase has demonstrated a significant difference from the D4 phase ($P = 0.007$). However, D16 phase also differed significantly from the D0 phase. ($P = 0.043$; Table 1) The diversity between the follicular and luteal stages also shows a significant difference ($P = 0.031$).

Table 1
Difference between Alpha diversity of four phases 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)	2.042	0.153	0.183
Diestrus (n=7)	Metestrus (n=9)	2.356	0.125	0.184
Diestrus (n=7)	Proestrus (n=8)	7.084	0.007	0.047
Estrus (n=9)	Metestrus (n=9)	0.001	0.964	0.965
Estrus (n=9)	Proestrus (n=8)	4.083	0.043	0.108
Metestrus (n=9)	Proestrus (n=8)	3.704	0.0542	0.108

Beta diversity between four phases 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 from two different farms were non-significant ($P > 0.05$) hence the further analysis was carried out using a cow as a unit of analysis. Beta diversity of D12 and D16 phases differed significantly from D4 ($p < 0.01$ and 0.023, respectively). Considering two stages i.e., follicular and luteal stages; of four estrous cycle phases shown has significant difference. Fig S2. Analysis of community structure using unweighted Unifrac distance showed differences between follicular and luteal samples ($P = 0.118236$). No significant differences were detected when using weighted Unifrac distance (Fig. S3C).

Taxonomical analysis

Data of taxonomic analysis was represented as percentage mean abundance of cow vaginal bacteria were found to belong to the two most abundant phyla *Proteobacteria*, and *Firmicutes* (Fig. 3A) which comprises of 94% bacterial diversity. Other less abundant phyla were *Actinobacteriota, Patescibacteria, Cyanobacteria, Bacteroidota, Verrucomicrobiota* and *Desulfobacterota* (Fig. 3A). Only *Bacteroidota, Firmicutes, Proteobacteria*, and *Verrucomicrobiota* revealed significant differences across the estrous cycle stages (Fig. 4). The D12 phase has a 1.2% mean proportion of *Bacteroidota* and a 40% mean proportion of *Firmicutes* which is higher than the other estrous phases (D0, D4 and D16). *Proteobacteria* is present across all the phases but the D12 phase has a lower mean proportion compared to other estrous cycle phases (Fig. 4). At the phylum level, the D12 phase shows significantly different diversity compared to other phases. But when the follicular (D16 and D0) and luteal (D04 and D12) stages were compared there was significant difference between phyla ($P = 0.025$). At the genus level analysis showed that the D12 phase showed a greater level of *Bacillus* (13% Mean population) compared to other groups (Fig. 3B). Estrus (D0) phase also showed greater *Bacillus* compared to that of the other two phases D4 and D16. At D 12 phase *Alcaligenes* (1.3% Mean abundance) is also greater than D16 and D4 phases. Metestrus (D4) phase 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 phase (Fig. 5). *Micrococcus, Stenotrophomonas*, UGC-010, *Massilia*, & *Methylobacillus* genus and

Pseudomonas genus was observed statistically significant at luteal stage and in the follicular stage, respectively (Fig. 6).

Hormone analysis

During D0, D4, D12 and D16 phases of estrous cycle progesterone levels were 0.39 ± 0.16, 0.78± 0.21 1.46± 0.82 and 3.86 + 1.44 ng/ml, respectively. No difference were observed between progesterone levels of D0 and D4 phases however, progesterone levels of D0 and D4 differed from D12 and D16 of estrous cycle($P < 0.001$).

Discussion

The present study demonstrated microbial diversity of *Bos indicaus* (Gir) cow during the estrous cycle under Indian conditions. Our study revealed a high diversity of bacteria and a limited diversity of Archaea. Cows from two farms were included in this report, although there was no difference in taxonomic analysis between the two farms ($P=0.3516$). This may be due to close proximity of farms and optimal feeding and management practices. There are reports (Quereda et al. 2020) on cow microbiota during the estrous cycle, however, are from herds of North America or Europe. In our study, we have analysed data from four different phases (D0, D4, D12, and D16) of estrous cycle, which are further investigated at stage level: follicular (D16 and D0) and luteal (D4 and D12). There are many factors which may influence the microbial diversity of vaginal microbiota including postpartum infections (Coleman et al. 1985).The microbiological diversity across the phase may be influenced by reproductive hormone concentrations presents during the estrous cycle (Mahesh, et al. 2020, Mahesh, et al. 2021). Progesterone, on the other hand, has been reported to colonise the vaginal microbiome and enhance bacterial diversity. Similarly, our study found that hormonal shifts had a considerable impact on microbial diversity during the four estrous phases. According to (Quereda, et al. 2020), the vaginal microbiota of cows was statistically different at the follicular and luteal stages of the estrous cycle in dairy heifers, which supports our findings that the follicular stage of the estrous cycle has much more variety than the luteal stage. (Fig. 2B). Finally, except for the diestrus phase, the principle coordinate plot (Fig. 7) demonstrates a greater commonality in microbial diversity. In this analysis, there is a substantial difference in alpha and beta diversity between the Luteal and Follicular stages. No significant phyla were observed, however, *Micrococcus, Stenotrophomonas*, UGC-010, *Massilia, Methylobacillus*, and *Pseudomonas* genus was observed statistically significant in the Luteal and follicular stages. Only the diestrus phase of the luteal stage reveals a statistically crucial distinction in the microbiota abundance. However, *Lactobacillus* is the most abundant *Firmicutes* genus in women vaginal microbiota, this genus is not abundant in the other mammalian species (Mehta et al. 2020, Ravel et al. 2011). Although in other mammals, lactic acid bacteria does not dominate as vaginal microbiota (Reid et al. 1985, White et al. 1989).

Our results on levels of progesterone hormone during estrous cycle demonstrated significantly lower level during D0 then D12 phase which may affect the microbial diversity in the vaginal environment. As diversity analysis also indicates that D12 phase shows maximum diversity among the all stages of

estrous cycle which can be influenced by the progesterone level. Also, alpha diversity and Beta diversity statistical data suggest the same. Though shift of principal microorganisms occurs during the bovine's ovarian cycle (Miller et al. 2017). However, the most mentioned genera of bovine's vagina LAB are *Lactobacillus, Pediococcus*, *Leuconostoc*, and *Weissella*, which are phylogenetically close to each other (Miller et al 2017) and usually measured together as the Lactobacillus group. Women's vaginal microbiota mainly consist of *Lactobacillus* genus (Mehta, et al. 2020, Swartz et al. 2014), however, bovine shows a low abundance of Lactobacillus genus (Mahesh et al. 2020, Mahesh et al. 2021), and shows high diversity of the phyla *Proteobacteria* and *Fermicutes*. Fig. S1 depicts bacterial changes in the lactobacillae family during the four phases of the estrous cycle. While Lactobaillus bacteria are found in the cow's vaginal tract, they can help to prevent or treat urogenital infections (Cribby et al. 2008). Quereda et al. (2020) observed that *Lactobacillus* is not dominant in the microflora of cow heifers which supports the results of our study. Swartz et al. (2014) presented that the most profuse genera were *Streptobacillus* spp. and *Aggregatibacter 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. This was in relation to previously findings on the core bovine uterine microbiota and postpartum vaginal microbiota (Laguardia-Nascimento et al. 2015). *Firmicutes* and *Proteobacteria* consist of 94% of the phyla of the vaginal diversity during the estrous cycle. Swartz et al. (2014) presented those Ewes and cow were predominantly colonized by the *Proteobacteria, Bacteroidetes*, and *Fusobacteria.* So many reports are supporting the results of our study. Giannattasio-Ferraz et al. (2020) 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), cattle phyla predominant by Firmicutes (~40–50%), Bacteroidetes (~15–25%), and Proteobacteria (~5–25%; Carmina et al. 2014). Ault et al. (2019) discovered that *Verrucomicrobiota* is abundant in postpartum cows, with a higher percent abundance in cows that were pregnant after insemination compared to cows who did not become pregnant. Similarly, they also 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. Similar genera were discovered in lower abundance in the findings of Quereda et al. (2020), which validates our findings. 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_Morganellacea. Micrococcus. Stenotrophomonas*, UGC-010, *Massilia, Methylobacillus* genus was observed statistically significant at luteal stage and *Pseudomonas* genus in the follicular stage.

Composition of the bacterial communities studied here were highly heterogeneous between different phases of the estrous cycle and their anatomical and physiological differences may influence microbiota

and reproductive tract (Laguardia-Nascimento, et al. 2015). Unlike some authors' hypothesis that hormonal maturity has a significant impact on the development of vaginal microbiota in cattle, the results obtained in this study demonstrate that hormonal maturity seems to have significant impact on the establishment of vaginal microbiota in cattle.

Conclusions

The most abundant bacterial phyla in the vaginal microbiota of Gir dairy 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 Gir dairy cows. Lactobacillus was not one of the most common genera found. Identification of bacterial communities during different phases 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.

Declarations

Compliance with ethical standards

Ethics approval

All experimental designs and protocols were approved by the Animal Ethics Committee of the Kamdhenu University and were under the recommendations of the academy's guidelines for animal research.

Declaration of interest

The authors declare that they have no competing interests.

Data and model availability statement

All data generated or analysed during this study are included in this published article (and its supplementary information files).

Contributions of Authors

The experimental scheme was designed by **VS, MJ**, and **CJ**. **VS**, 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.

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Figures

Figure 1.

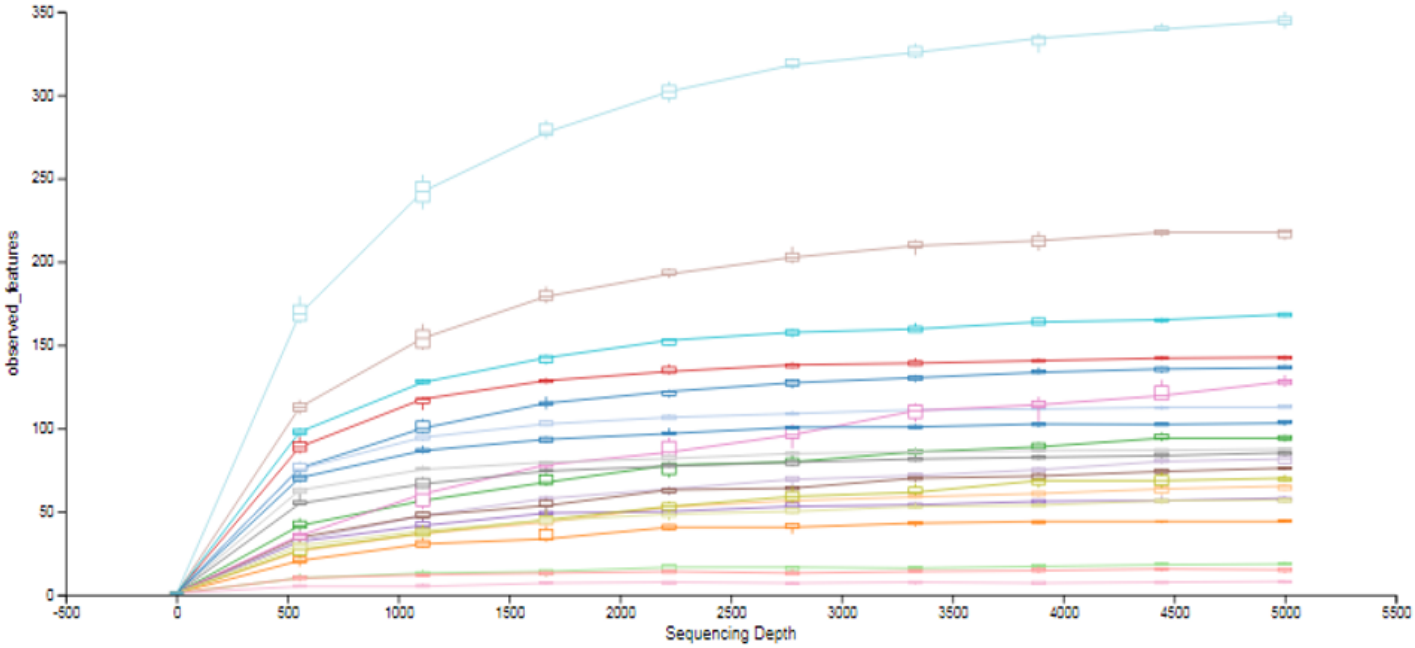

Figure 1

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

Figure 2.

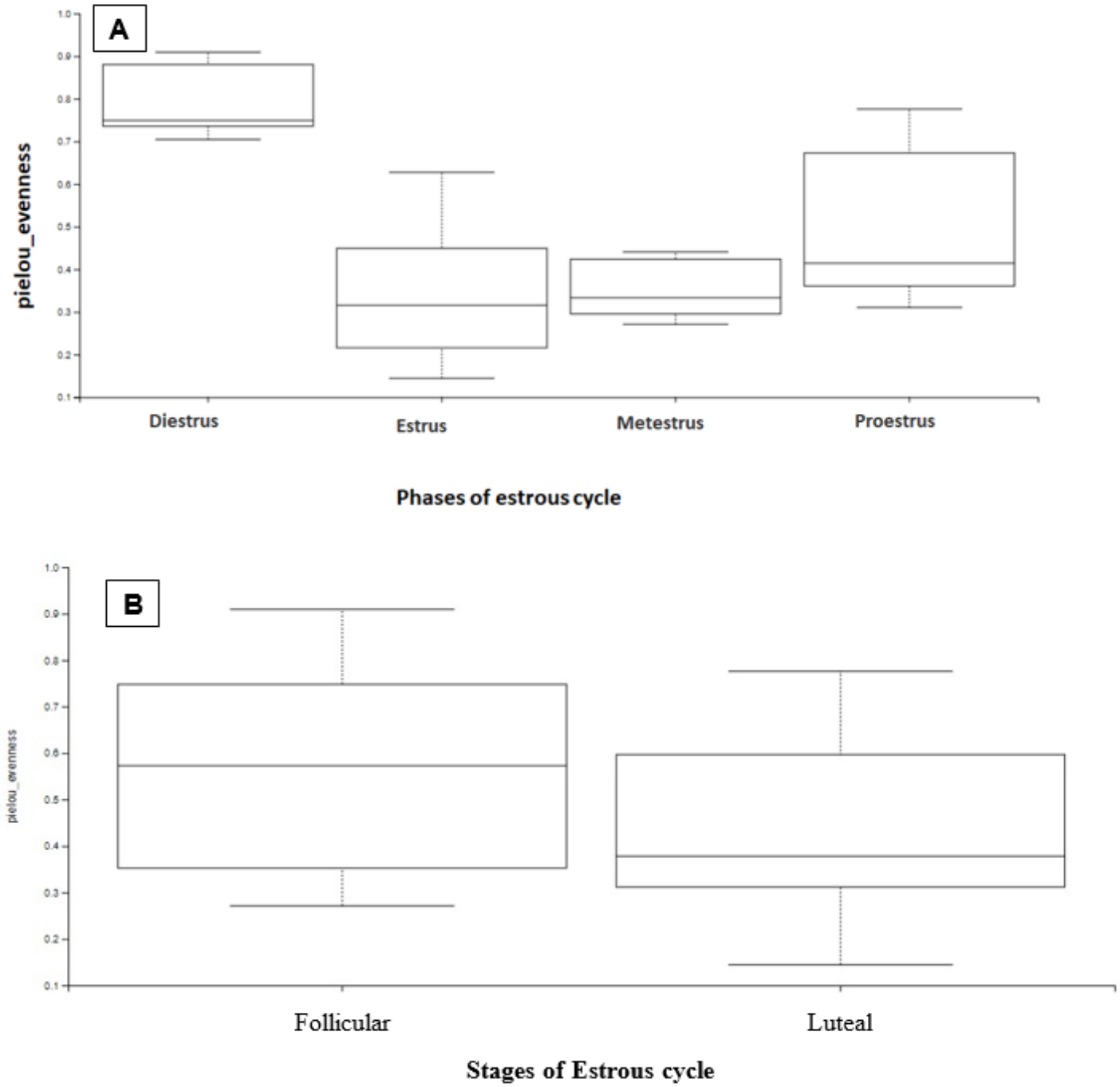

Figure 2

A. Alpha Diversity box plot of all the phases at sequence depth of 5000 and B. shows richness of vaginal microbial communities in follicular and luteal phases.

Figure 3A

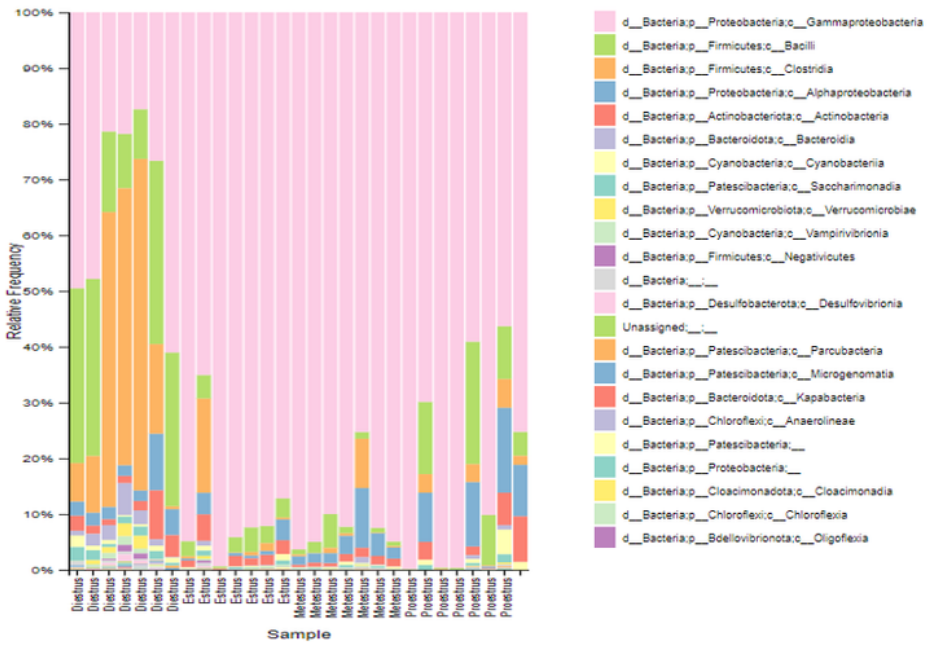

Figure 3B

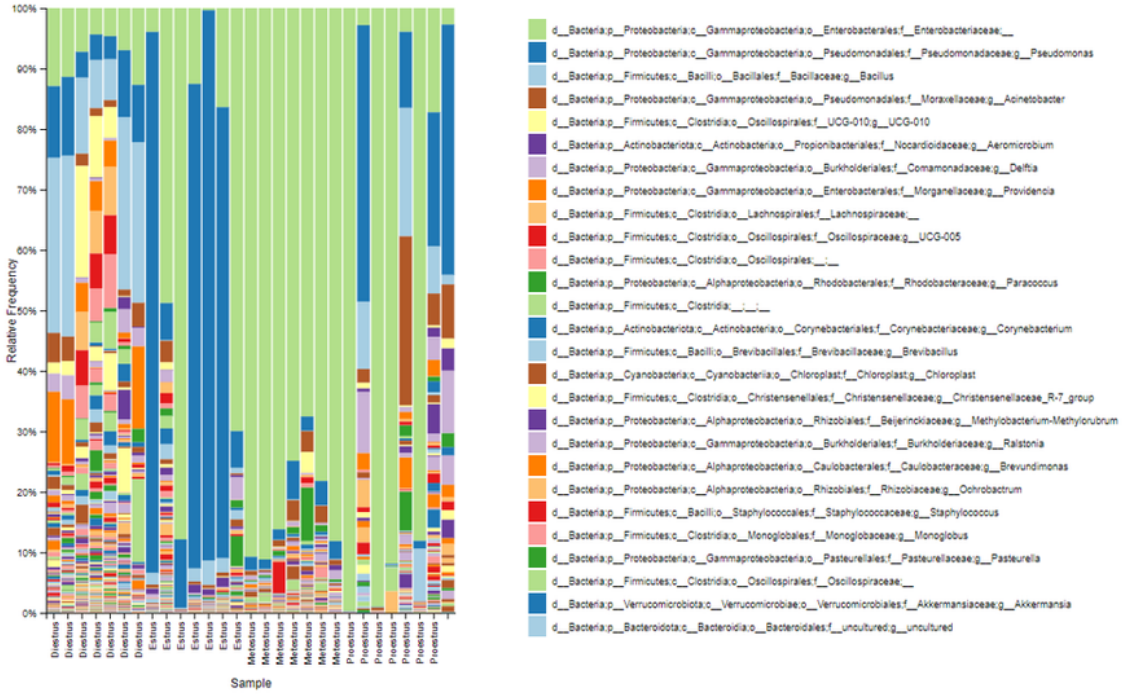

Figure 3

A. Beta Diversity of all the estrous cycle phases at Phylum Level in cows. At the genus level analysis showed that the D12 phase showed a greater level of Bacillus (13% Mean population) compared to other groups (Fig. 3B)

Figure 4

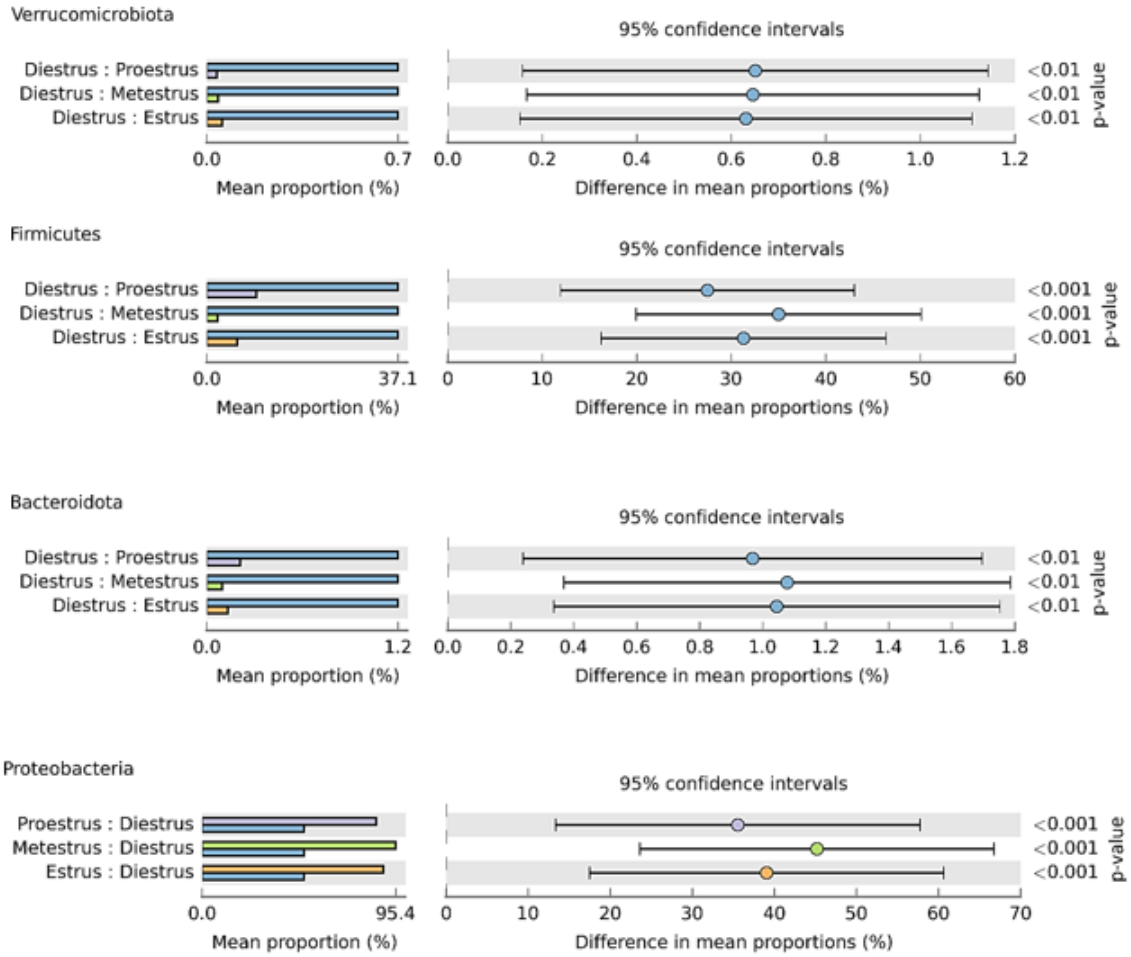

Figure 4

Significant phyla after statistical analysis using multiple test ANOVA and statistical correction done using Benjamini-Hochberg FDR of data Beta Diversity of all the phases at Phylum Level

Figure 5

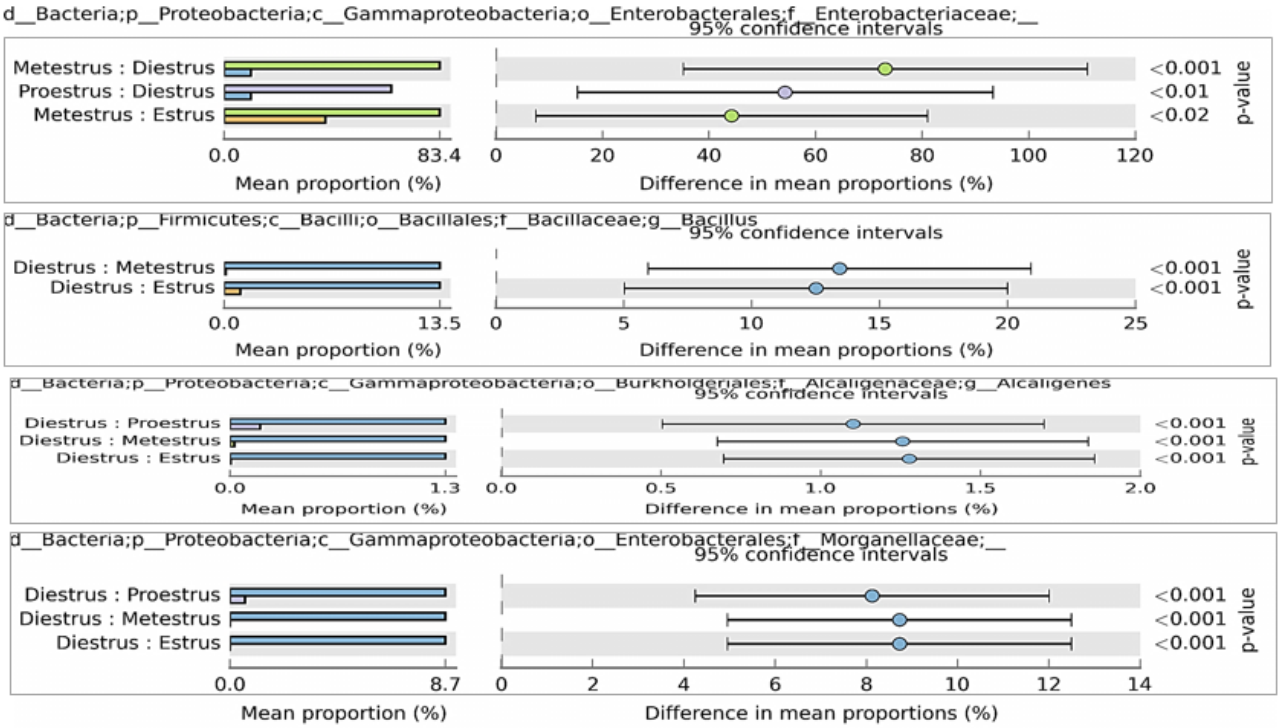

Figure 5

Significant Genus after statistical analysis using Multiple test ANOVA and statistical correction was done using Benjamini-Hochberg FDR of data Beta Diversity of all the phases at Genus Level

Figure 6

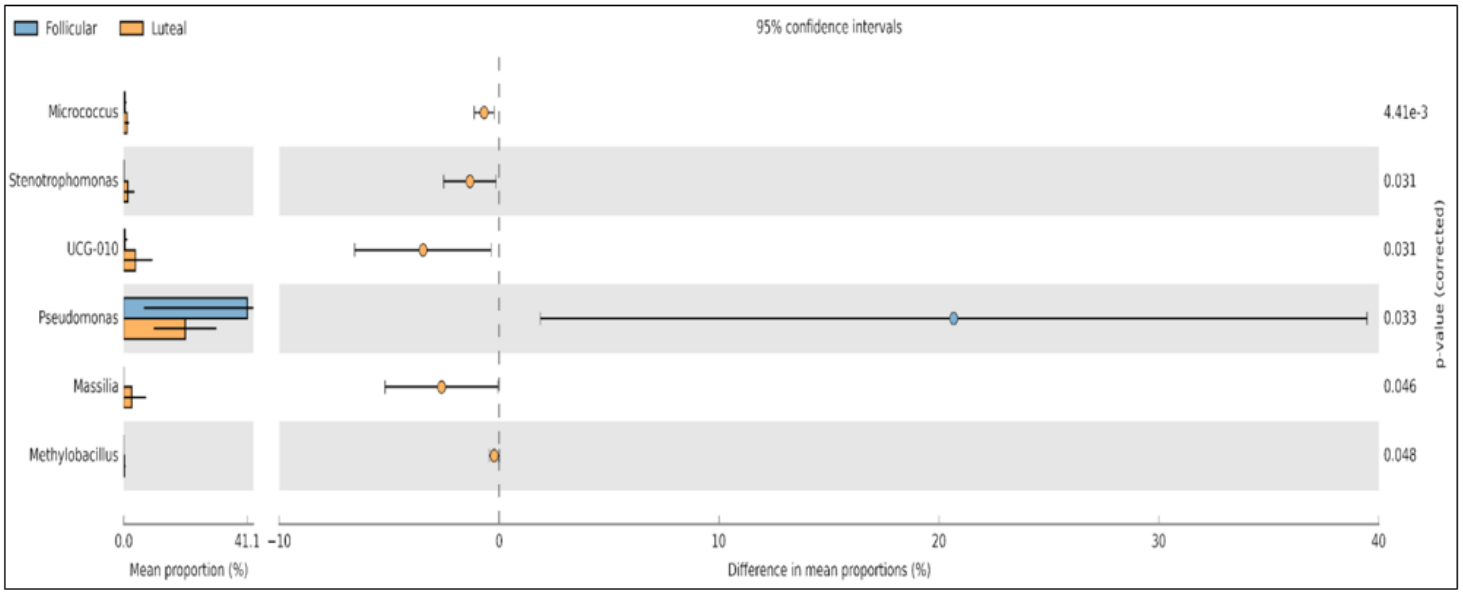

Figure 6

After statistical analysis using Multiple test ANOVA without statistical correction, the genus was found to be significant for the follicular and luteal stages.

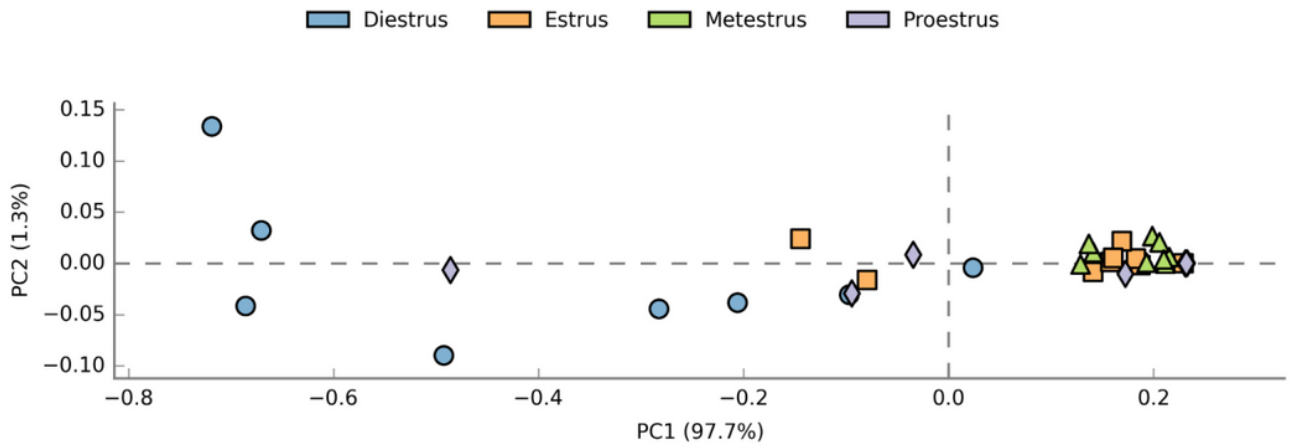

Figure 7

Principal component analysis (PCA) plot analysis of different phases of estrous cycle

Supplementary Files

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