RESEARCH ARTICLE

Vaginal Microbiota during Estrous Cycle and its Plausible Association with Certain Hematological Parameters in *Bubalus bubalis*

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Abstract

The study on the dynamics of vaginal microbiota during the estrous cycle and its plausible association with certain hematological parameters in *Bubalus bubalis* was conducted on nine buffalo heifers at a private dairy farm. Out of nine, eight heifers responded to the synchronization protocol and included in the experiment. Blood collection using vacutainers and vaginal flushing using sterile normal saline with a syringe and AI sheath was carried out on '0' (estrus), 4th (metestrus), 9th (diestrus) and 19th day (proestrus) of the synchronized estrous cycle. The collected vaginal samples were lyophilized, DNA isolated using Qiagen stool kit, and amplified with fusion primer for Bacteria (16S rRNA), Fungi (ITS 6-8), and Archaea (16S rRNA). The amplicon library constructed was sequenced through Ion PGM machine and annotation was carried out through MG-RAST with rRNA database SILVA ssu with 98% identity. The results revealed four major domains - Bacteria (93.8%), Eukaryota (5.7%), Archaea (0.006%), and Unclassified sequences (0.41%). A total of 2196 bacterial species, 17 fungal species, and two archaeal species were detected confirming the richest diversity of vaginal microflora in buffalo heifers. The highly abundant domain-bacteria contained 18 bacterial phyla and were grouped into four high (>5%), six moderate (0.1-5%), and eight less abundant groups (0-0.1%). The results of hematological parameters were within the normal range. There was no impact of four stages of the estrous cycle on hematological parameters studied and no associations of bacterial phyla with them. Out of a total of 588 bacterial genera, only 14 showed a negative association, while four genera showed a positive association with blood monocytes. Similarly, only Moraxella, Microcoleus, Thiomonas showed negative associations with WBC, lymphocyte, and granulocyte, and Weissella genus showed a negative association with lymphocyte. No association was observed between types of white blood cells and fungus or archaea. These results of association of vaginal microflora with hematological parameters may be of unknown reasons and suggest further investigations.

Keywords: Association, Buffalo, Estrous cycle, Hematology, Vaginal microbiota.

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Introduction

Buffalo has emerged as black gold for farmers of the Indian milk industry, which contributes about 49.0% of total milk production (BAHS, 2019). However, per capita, milk production of buffalo is still a challenge (Maurice Landes *et al.,* 2017; Kumar *et al.,* 2018). The economic viability of keeping buffaloes depends upon normal reproduction. The microbial diversity, its impact, role, and dynamics during different physio-pathological conditions of the vagina and uterus of cows have been studied in depth (Bicalho *et al*., 2017, Ault *et al.,* 2019, Galvão *et al.,* 2019). It has been well documented that the vagina of several mammalian species has a mixed microbial flora consisting of aerobic, strict anaerobic and facultative anaerobic organisms. Mahalingam *et al.* (2019) and Mahesh *et al*. (2020) demonstrated the diversity of vaginal microbiota and difference in abundance level of Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Tenericutes during proestrus, estrus, diestrus and metestrus phase of the estrous cycle of buffaloes.

Using culture-based techniques, it has been well documented that micro-organisms play a significant role in deciding the fertility of cattle and buffaloes (Sheldon and Dobson, 2004; Sheldon *et al.,* 2014, Saraswat and Purohit, 2016). Interestingly, sex steroid hormones, physio-

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pathological conditions, environment, nutrition has an impact on the reproductive microbiome in bovines. However, the interrelationship between host reproductive microbiome and hematological parameters has not been explored much. The role of the hematopoietic system in transporting endocrine hormones to the targeted site for the smooth functioning of reproduction and WBCs/DLCs in the defense mechanism of a body is well established. Recently we demonstrated the influence of estradiol and progesterone on the dynamics of vaginal microbiome (Mahesh *et al.,* 2020). However, no information is available on the plausible association between vaginal microbiome and certain hematological parameters during different stages of the estrous cycle. Hence, the present study was undertaken with the objective of studying vaginal microbiota during the estrous cycle and its plausible association with certain hematological parameters in *Bubalus bubalis*.

Materials and Methods

The study was performed on buffalo heifers from August to December 2016 at an organized dairy farm in village Motipura, Gandhinagar, Gujarat. All the animals were fed and managed optimally.

Animal Selection

Nine pubertal buffalo heifers weighing 262.25 ± 49.38 kg b.wt., and age 3.75 ± 0.55 years, and had experienced estrus at least once were included in the study. Initially, the animals were dewormed with Panacur-3g (MSD Animal Health, India) and were screened for Brucella, TB, and JD using a standard diagnostic methodology, and were found negative. No animal was treated with antibiotics for three months before the experimentation. The status of the reproductive organ was examined initially using ultrasonography (Easi-Scan, BCF Technology India Pvt Ltd, India). Irrespective of the palpation findings of ovary and uterus, nine buffalo heifers were subjected to standard Ovsynch protocol of estrus induction using i/m administration of buserelin acetate 20 µg (Receptal® MSD, India) on day zero and nine, and 25 mg dinoprost tromethamine (Lutalyse, Zoeits, India) on day seven. Eight buffalo heifers exhibited estrus within 79.0 ± 3.0 hours were included in the study.

Sample Collection

Blood samples from the jugular vein and vaginal flushing samples were collected simultaneously on the day of estrus (day 0), metestrus (day 4), diestrus (day 9) and proestrus (day 18) of the synchronized estrous cycle using 8 ml EDTA vacutainers and sterile AI sheath and syringe using 50 ml normal saline, respectively, and were transported to the laboratory at 4°C temperature. Before taking the vaginal samples, external genitalia was washed with 4% chlorhexidine (Excelle, DRE Veterinary, India). AI sheath was passed into the vaginal fornix after holding cervix per-rectally followed by administration of 50 mL sterile normal saline. Without disconnecting the syringe the plunger was drawn back to retrieve vaginal secretions along with normal saline. The sample containing vaginal secretions was transferred equally in two 50 ml centrifuge tubes and stored at -80°C until further use. Hematological analysis of blood samples for TLCs and DLCs was carried out using Exigo blood analyzer (Boule Diagnostics, India).

Nucleic Acid Extraction and Amplification, Library Preparation and Sequencing

The stored vaginal flushing samples were thawed at room temperature and subjected to lyophilization followed by DNA isolation using the Qiagen Stool Kit (Qiagen India PVT. Ltd. New Delhi, India). The presence of DNA was evaluated with 0.8% agarose gel electrophoresis. Four Samples did not show an evidently required quantity of DNA extraction and hence were dropped from the analysis. Therefore the total 28 samples out of 32 were amplified using three different fusion primers for bacteria, archaea, fungi, and 84 amplicon libraries were formed. Similar barcode and adapter tag were used in fusion primers for amplification of bacteria, archaea and fungus.

Amplification of bacteria was carried out with 16s universal primers with standard procedure. Amplification of fungi was carried out using ITS-6 5′ to 3′ CGATTCCGTAGGT GAACCTGCGG and ITS-8 5′ to 3′ GCACATCGATGAAGAACGCT primers as described by Cooke *et al*. (2000). Amplification of archea was carried out as per Laguardia-Nascimento *et al*. (2015) using 300 fEyAr 5′′ to 3′′ AGCRRGAGCCCGGAGATGG and 954 rEyAr 5′′ to 3′′ CGGCGTT GARTCCAATTAAAC primers. The amplified products were used as a template for PCR with fusion primers. The metagenomic library was constructed using the Qubit dsDNA HS Assay Kit with Qubit 2.0 Fluorometer. ISPs were prepared using ion One touch machine according to the manufacturer's protocol. Sequencing was carried out using Ion PGM™ sequencer (Thermo Fisher Scientific Pvt. Ltd., India).

Data Processing and Analysis

The demographic and reproductive data of experimental animals were documented in excel spreadsheets. The data obtained through NGS sequencing of 28 buffalo-vaginal samples were uploaded into the online server MG-RAST. To increase the accuracy of results in RNA database analysis, the identity was fixed to 98%, e value 10^{-8} and length 80 bp, even though minimum criteria given by the MG-RAST is identity 60%, e value 10^{-5} and length 15 bp. All the results were analyzed on STAMP by one way ANOVA with Tukey-Kramer post hoc test with $p < 0.05$. The impact of phases on hematological parameters was analyzed using one way ANOVA followed by a post-hoc test. The association between buffalo vaginal microbiota with hematological parameters was studied using Pearson's correlation in SPSS® software (SPSS 26, IBM Pvt Ltd, Bengaluru, India).

Results and Discussion

The results of the rarefaction curve of all 28 sequenced libraries are demonstrated in Fig. 1. The rarefaction curve is used for demonstrating the species richness of the sample. This curve grows rapidly at first, where each read in the sample identifies as a new organism and slowly starts to plateau when the rare species remain to be sampled. If the rarefaction curves for the samples reach the plateau, this suggests you have a good representation of the microbial community as most of the abundant species are represented with some rare species (Gotelli and Chao, 2013). The result of 28 samples in this study shows satisfactory representation and all the curves reached a plateau, which indicates that the environment was adequately explored, giving true representation of the operational taxonomic unit (OTU). In this study, vaginal microbiota of the buffalo, heifers consisted of four major domains, *viz.,* bacteria, archaea, eukaryota, and unclassified sequences. In all 2196 bacterial, 17 fungal and two archaeal species were identified, which confirm that buffalo vagina has a rich diversity of microflora compared to other species. The details on these results were reported somewhere else (Mahesh *et al*., 2020).

The vaginal microbiota of the buffalo heifers was grouped into four major domains in which bacteria accounted 93.8%, eukaryotes 5.7%, followed by archaea 0.4 % and the unclassified sequences were 0.006 %. In the present study,

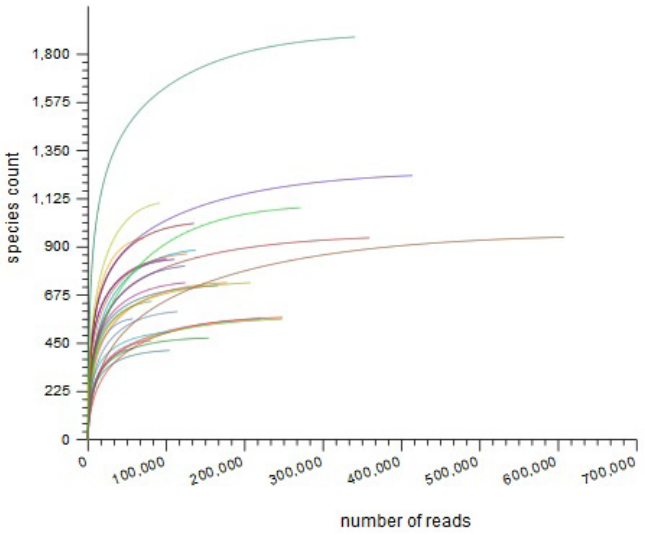

Fig. 1: Rarefaction curve of 28 libraries of vaginal samples collected during estrous cycle of eight buffalo heifers

a total of 2196 bacterial species, 17 fungal species and two archaeal species were identified. The variability observed within each group was remarkable. This represents the absolute abundance of all the four biological domains. The bacterial domain contained a total of 18 bacterial phyla, which were grouped into high, moderate, and less abundant groups. Irrespective of stages of estrous cycle, the four most highly abundant phyla were Firmicutes, Actinobacteria, Proteobacteria, and Unclassified Bacteria with relative abundance >5%. Thermotogae, Bacteriodetes, Fusobacteria, Dienococcus-Thermus, Cyanobacteria, Teniricutes were the six phyla grouped into moderately abundant with relative abundance 0.1-5%. Verrucomicrobia, Synergistetes, Spirochaetes, Chloroflexi, Acidobacteria, Nitrospirae, Chlorobi, Fibrobacteres were grouped into less abundant phyla with a relative abundance 0-0.1%. In the vagina of buffalo (Mahalingam *et al.,* 2019) and Nellore cattle (Laguardia-Nascimento *et al.,* 2015), phyla Firmicutes, Proteobacteria, and Bacteroides were reported as abundant groups.

Eukaryota contributed about 5.7% of overall vaginal microbiota in the present study. Among the most eukaryotes present in the vaginal tract of buffalo heifers, Streptophyta accounted for 81.4%, Arthropoda 7.7%, Ascomycota 5.02%, Nemetoda 3.06%, Unclassified fungi 3%, Bacillariophyta 0.3%, Chlorophyta 0.04%, Unclassified Eukaryota 0.02% and Cinidria 0.008 %. The two fungal phyla detected were Ascomycota and Unclassified fungi, which accounted for 5.3% of total Eukaryota. The abundance of Streptophyta, a plant genus, in the vagina may be due to the wallowing habit of buffaloes. Best of our knowledge and as per literature, this is the first study that has explored eukaryotes in buffalo vagina. Previously Laguardia-Nascimento *et al*. (2015) demonstrated eukaryotes in the vaginal tract of Nellore cattle; however in the present study, eukaryotes abundance was somewhat higher, maybe due to species difference.

The hematological parameters, *viz*., total WBC, lymphocyte, monocytes, and granulocytes during four stages of estrous cycle are documented in Table 1. There was no impact of four stages of estrous cycle on hematological parameters studied (p >0.05). The results were within the normal range as described earlier (Ellah *et al.* 2013). The non-significant associations of differential leucocytes counts obtained with high, moderate, and less abundant bacterial phyla are documented in Table 2. No association

Table 1: White blood cells (WBC), lymphocyte, monocyte and granulocyte profile during different stages of estrous cycle in buffalo heifers (n = 8; Mean ± SD)

Stage	*WBC* $\times 10^3/\mu L$	*Lymphocyte* $\times 10^3/\mu L$	*Monocyte* $\times 10^3/\mu L$	*Granulocyte* $\times 10^3/\mu L$
Estrus	6.63 ± 2.2	3.57 ± 1.5	0.66 ± 0.17	2.40 ± 0.7
Metestrus	8.03 ± 1.9	4.68 ± 1.4	0.70 ± 0.2	2.65 ± 1.4
Diestrus	7.40 ± 2.7	4.33 ± 1.3	0.53 ± 0.17	2.53 ± 1.4
Proestrus	7.36 ± 2.2	4.19 ± 1.8	0.63 ± 0.2	2.53 ± 0.9

NB: None of the parameters varied significantly between stages (p > 0.05).

Table 2: Association of high, moderate and less abundant bacterial phyla with leucocyte counts

	High abundant phyla (>5%)			
Leucocyte	*Firmicutes*	*Actinobacteria*	*Proteobacteria*	*Unclassified Bacteria*
WBCs	-0.399	0.224	0.474	0.599
Lymphocyte	-0.438	0.247	0.572	0.62
Monocyte	0.209	-0.128	-0.567	-0.081
Granulocyte	-0.397	0.224	0.459	0.598

	Moderate abundant phyla (0.1-5%)					
Leucocyte	*Thermotogae*	*Bacteroidetes*	*Fusobacteria*	*Deinococcuss*	*CyanoBacteria*	*Tenericutes*
WBCs	-0.844	0.753	0.59	-0.719	-0.413	0.04
Lymphocyte	-0.899	0.735	0.477	-0.761	-0.337	0.047
Monocyte	0.266	0.179	0.76	0.199	-0.51	-0.038
Granulocyte	-0.835	0.756	0.604	-0.715	-0.424	0.035

	Less abundant phya (0-0.1%)							
Leucocyte	*Verrucomicrobial*	*Synergistetes*	*Spirochaetes*	*Chloroflexi*	*Acidobacteria*	*Nitrospirae*	*Chlorobi*	*Fibrobacteres*
WBCs	0.67	0.689	0.871	0.382	0.943	0.044	0.307	0.846
Lymphocyte	0.761	0.743	0.789	0.521	0.897	0.167	0.452	0.834
MonoCyte	-0.509	-0.275	0.598	-0.835	0.372	-0.761	-0.879	0.147
Granulocyte	0.659	0.683	0.879	0.365	0.948	0.027	0.289	0.848

Note: None of the correlation was statistically significant at $p < 0.05$.

Table 3: Correlation of bacterial genera from the vagina with blood monocyte

Genus	*Monocyte*	*p-value*	*Genus*	*Monocyte*	*p-value*
Salmonella	-0.972*	0.028	Paracoccus	-0.964*	0.036
Sporosarcina	-0.975*	0.025	Acetobacter	0.963*	0.037
Microlunatus	-0.973*	0.027	Lechevalieria	-0.967*	0.033
Syntrophococcus	-0.998**	0.002	Hyphomicrobium	-0.978*	0.022
Arcobacter	-0.983*	0.017	Chromohalobacter	-0.965*	0.035
Shigella	-0.988*	0.012	Bordetella	0.987*	0.013
Kocuria	-0.965*	0.035	Peptostreptococcus	0.951*	0.049
Unclassified (Rhizobiales)	-0.991**	0.009	Unclassified (Vibrionaceae)	-0.966*	0.034
Unclassified (Rhodospirillaceae)	-0.967*	0.033	Brevundimonas	0.992**	0.008

**p<0.01, *p<0.05. NB: None of the Genera mentioned in above shown association with WBCs, lymphocyte and granulocyte ($p > 0.05$).

Table 4: Correlation of bacterial genera from vagina with WBC, lymphocyte and granulocyte

Genus	*WBC*	*P-value*	*Lymphocyte*	*P-value*	*Granulocyte*	*P-value*
Moraxella	-0.957*	0.043	-0.989*	0.011	-0.951*	0.049
Microcoleus	-0.969*	0.031	-0.996**	0.004	-0.964*	0.036
Thiomonas	-0.989*	0.011	-0.987*	0.013	-0.987*	0.013
Weissella	-0.969*	0.031				

**p <0.01, *p <0.05. NB: None of the Genera mentioned in table shown an association with monocyte ($p > 0.05$).

of hematological traits was found at phyla level ($p > 0.05$), but at genera level certain associations were significant. However, out of 588 genera identified, only a few had a significant association with certain hematological attributes (Tables 3 and 4). The use of leucocyte count to determine endometritis in cattle and buffalo is a common manifesting role of WBCs during infection. This study evident that there is no impact of four stages of estrous cycle of healthy buffalo heifers. However, results show an association of genus with WBCs, which require further investigation. The result revealed that the WBC counts were in its normal range; however, showed association at the genus level. Many plausible factors like a defense mechanism, immune response, neuroendocrine response, etc. might affect the association. Therefore the result of this study warrants further investigation.

Out of total 588 genera obtained, only 14 bacterial genera, *viz.*, Salmonella, Sporosarcina, Microlunatus, Syntrophococcus, Arcobacter, Shigella, Kocuria, unclassified Rhizobiales, Unclassified Rhodospirillacae, Paracoccus, Unclassified Vibrionaceae, Lechevalieria, Hyphomicrobium and Chromohalobacter showed negative correlation ($p < 0.05$) with blood monocytes, while Bardotella, Peptostreptococcus, Acetobacter, and Brevundimonas genera showed a positive correlation ($p < 0.05$; Table 3). Similarly, only Moraxella, Microcoleus, Thiomonas showed negative correlations with WBC, lymphocyte, and granulocyte ($p < 0.05$; Table 4). Weissella genus showed a negative correlation only for WBCs

(Table 4). Results on associations of vaginal microbiota with differential leukocyte count and total WBC is of unknown reasons and warrants further such studies in the future. At the different phylogenetic levels of Archea or Fungi, no association was revealed ($p > 0.05$). This might be due to low abundance as compared to bacterial abundance in the vagina. Our results warrant further studies to explore the association and role of hematological parameters and other immune responses with an abundance of microbiome of the vagina and/or reproductive system in animals.

Conclusions

The vaginal microbiota of the buffalo heifers consisted of four major domains *viz.,* bacteria, archaea, eukaryota, and Unclassified sequences. Bacteria accounted 93.8%, eukaryotes 5.7%, archaea 0.006% and Unclassified sequences 0.41%. Out of total 2196 bacterial species, 17 fungal species and two archaeal species were identified, which confirms that buffalo vagina has a rich diversity of microbiota. Indeed the study demonstrates rich diversity of microbiota of vagina in buffalo heifers. The study revealed some association between certain hematological parameters with different genera, which warrants further research to justify the results.

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