RESEARCH ARTICLE

Dynamics of Vaginal Microbiota during Estrous Cycle and its Association with Reproductive Hormones in *Bubalus bubalis*

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Abstract

This experiment aimed to evaluate the dynamics of vaginal microbiota during different estrous cycle stages in cyclic buffalo heifers using metagenomic analysis and its association with estradiol (E_2) and progesterone (P_4) hormones. Out of nine buffalo heifers, eight responded to ovsynch protocol were included in the trial. Vaginal flushing was carried out using the assembly of sterile normal saline with a syringe and AI sheath on day 0 (estrus), day 4 (metestrus), day 9 (diestrus), and day 18 (proestrus) of the estrous cycle followed by blood sample collection. The vaginal flushing was lyophilized, DNA were isolated and amplified with Phusion primer for bacteria (16S rRNA), fungus (ITS 6-8), and archaea (16S rRNA). The amplicon library was constructed, sequenced, and annotated through MG-RAST server with RNA database SILVA ssu with 98% identity. The results of the study revealed four major domain- bacteria (93.8%), eukaryota (5.7%), archaea 0.006% and unclassified sequences (0.41%). A total of 2196 bacterial species, 17 fungal and two archaeal species, were detected, confirming the rich diversity of vaginal microflora in buffalo heifers. The highly abundant domain of bacteria contained 18 bacterial phyla, which were grouped into four high (>5%), six moderate (0.1-5%) and eight less abundant groups (0-0.1%); and it contained 589 genera of which Amycolatopsis, Cardiobacterium, Cellulomonas, Pimelobacter, Rickettsia, Promicro-monospora, Xanthobacter, Anerotruncus, Desulfonispira, Leptospira and Massilia were significant. The two fungal phyla detected were Ascomycota and unclassified fungi. Out of 589 bacterial genera, 48 genera showed positive correlation and four genera showed negative correlation with plasma P_4. The results of fold change in Log2 values and association of E_2 and P_4 hormones with high and less abundant phyla and genera of bacteria during four phases suggest dynamics of vaginal microbiota during the estrous cycle. Though the present study demonstrated novel findings and is the first of its kind in buffalo heifers, it warrants further research.

Keywords: *Bubalus bubalis,* Estrous cycle, Reproductive hormones, Vaginal microbiota.

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Introduction

Recent studies in cattle demonstrated the in-depth role of microbiota during different physio-pathological conditions of reproduction in cattle (Bicalho *et al*., 2017; Ault *et al*., 2019; Galvão *et al*., 2019); however, limited information is available for buffaloes (Mahalingam *et al*., 2019). Using culture-based techniques has been well proved that microorganism plays a significant role in the fertility of cattle and buffaloes (Sheldon and Dobson, 2004; Sheldon *et al*., 2014; Saraswat and Purohit, 2016). It has also been known that vaginal microbiota can invade the uterus through the cervix, which is open during parturition and estrus (Sheldon *et al*., 2014; Galvão *et al*., 2019). Thus, microorganisms in the vagina pose a potential to decide the fertility of the cows and buffaloes (Kumar *et al*., 2011; Saraswat and Purohit, 2016). The limitations of isolating specific organisms (1%) using culture-based techniques are eliminated using culture-independent molecular approaches and open a new horizon to look at unexplored microbiota (Hugenholtz *et al*., 1998; White *et al*., 2011).

Interestingly, sex steroid hormones participate in the communication between microorganisms and mammalian hosts (García-Gómez *et al*., 2013; Neuman *et al*., 2015) and modulate metabolism, growth, or virulence pathogenic bacteria (Wessels *et al*., 2018). These evidences suggest that sex steroid hormones play a key role in the modulation of bacterial-host interactions (García-Gómez *et al*., 2013; Garcia-Grau *et al*., 2019). Recently using high-throughput amplicon sequence of 16S RNA gene, Mahalingam *et al*. (2019) and

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Patel (2019) demonstrated the diversity of vaginal microbiota and difference in abundance level of Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Tenericutes during different phases of the estrous cycle of buffaloes. They hypothesized that physiological or biochemical processes could determine microbial composition during proestrus, estrus, metestrus, and diestrus. This is the only information available on buffalo vaginal microbiota during an estrous cycle to the best of our knowledge. Nevertheless, there is no documentary evidence available on the association of sex hormones with dynamics of vaginal microbiota (bacteria, eukaryota, fungus, and archaea) during the estrous cycle of buffaloes that attended puberty. Hence, considering the dearth of information in this area, the objective of this research was to study the dynamics of vaginal microbiota during the estrous cycle and its association with reproductive hormones in *Bubalus bubalis*.

Materials and Methods

The present study was conducted on buffalo heifers from August to December 2016 at an organized private dairy farm in village Motipura, Gandhinagar, Gujarat, India. The study was approved by Institutional Animal Ethics Committee of Kamdhenu University.

Animal Selection

Nine pubertal buffalo heifers which had estrus at least once between 3 to 4.5 years with bodyweight from 210 to 420 kg were included. Animals were fed *ad libitum* with wheat hay, lucerne, and green grass. The concentrate mixture (cottonseed, groundnut, soybean, maize grains etc.) was fed @ 2 kg per animal after boiling. Animals were dewormed with Panacur-3g (MSD Animal Health, India) and screened for Brucella, TB, and JD using standard diagnostic methodology before starting the experiment and were found negative. No animal was treated with antibiotics three months prior to the experimentation. Before starting the experiment, the status of the reproductive organ was examined using ultrasonography (Easi-Scan, BCF Technology India Pvt. Ltd. India) and recorded in evolved proforma for data recording. Irrespective of the palpation findings of ovary and uterus, nine buffalo heifers were subjected to synchronization protocol; initiated with intramuscular administration of buserelin acetate 10 µg on day zero followed by 25 mg dinoprost tromethamine (Lutalyse, Zoeits India) on day seven. Eight heifers experienced estrus within 72 hours and were included in the metagenomic study.

Sample Collection from Animals

Vaginal flushing and blood samples were collected on the day of estrus (day 0), metestrus (day 4), diestrus (day 9), and proestrus (day 18) of the synchronized estrous cycle. Before taking the vaginal samples, external genitalia was washed with 4% chlorhexidine. The assembly preparation for sample collection was as per Mahesh *et al.* (2020). The sample containing vaginal secretions was transferred equally in two 50 mL centrifuge tubes and transported, maintaining 4°C temperature to be stored at -80°C until further use.

Blood samples were collected 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 2 ml plasma storage vials at -20°C until analyzed for estradiol (E2) and progesterone (P4) concentration by Chemiluminescence immunoassay CLIA method in a commercial laboratory. The part on the association of vaginal microbiota with hematological parameters were shown somewhere else (Mahesh *et al*., 2020).

Hormonal Assay

A hormonal assay was performed from a plasma sample using Elecsys® Estradiol III and Elecsys® P_4 III by the Cobas e-411 analyzer (Roche Diagnostics, Germany). Cobas e-411 analyzer uses chemiluminescence immune assay for the analysis of the E_2 (pg/mL) and P_4 (ng/ml) concentrations. The intra and inter assay coefficient of variation for E_2 was 9.62 % and 9.8 %, and for P_4 was 8.7 % and 8.8 %, respectively.

Nucleic Acid Extraction and Amplification

The stored vaginal flushing samples were subjected to lyophilization and stored at room temperature to be used for further DNA isolation. DNA was isolated from the lyophilized sample using the Qiagen Stool Kit (Qiagen India Pvt. Ltd. New Delhi, India) with slight modification in the manufacturer's protocol in data analysis and the presence of DNA was checked with 0.8% agarose Gel electrophoresis.

DNA isolated was subjected to specific amplification with bacterial phusion primers, while archaea and fungus samples were initially amplified with normal primers and the same amplicon was subjected to further amplification with the phusion primers (Mahesh *et al.,* 2020). Each sample (n=28) was amplified using three different phusion primers (Bacteria, Archaea, Fungus), and the amplicon library (n=84) was formed. One set each, *i.e.,* 24 similar Barcode and Adapter tag, was used in phusion primers for amplification of bacteria, archaea and fungus. Amplification of bacteria was carried out with 16S universal primers, for each sample, the primer with different adapter and barcode was used. Amplification of fungus was carried out using ITS-6 5′ to 3′ CGATTCCGTAGGTGAA CCTGCGG and ITS-8 5′ to 3′ GCACATCGATGAAGAACGCT primers (Cooke *et al*., 2000). Amplification of Archaea was carried out with 300fEyAr 5″ to 3″ AGCRRGAGCCCGGAGA TGG and 954rEyAr 5″ to 3″ CGGCGTTGARTCCAATTAAAC primers (Laguardia-Nascimento *et al*., 2015). The amplified products were used as a template for PCR with phusion primers. The processing of samples for

nucleic acid extraction and amplification of Bacteria, Archaea and Fungus DNA was done as per Mahesh *et al*. (2020)

Metgenomic Libraries Construction and Sequencing

The PCR amplicons of bacteria and fungus (approx. 20 µL) in non-sticky Eppendorf tube were added with 1.5 times Agencourt reagent, *i.e.,* 30 µL, vertexed, whereas the PCR amplicon of archaea was added with 108 µL Agencourt reagent pipetted up and down, and allowed at room temperature for 5 min. The sample was given a short spin and arranged in Dynal magnetic rack, and kept for 3 minutes until the solution becomes colorless. The sediment was carefully pipetted out without disturbing the magnetic beads which hold the amplicon. The same Eppendorf tube containing magnetic beads was added with 500 µL 70% ethanol. It was rotated 2-3 times so that beads move towards the rack and get purified; later ethanol was carefully pipetted out discarded. The same step was repeated once again, Eppendorf tube was kept 5 min for air drying in a same magnetic rack and after a short spin to remove the traces of ethanol. The amplicon attached in the magnetic beads was detached by adding Low TE 20 µL for bacterial and fungal amplicons and Low TE 25 µL for archaea with proper vertexing and spin. The Low TE-containing amplicons were aspirated into 1.5 ml Eppendorf tubes, which formed purified amplicon libraries. Amplicon quantitation was performed using Qubits DNA HS Assay Kit with Qubit 2.0 Flurometer.

After quantifying the library with Qubit assay, molar concentration was calculated, and library was diluted to 100 pmol with Low TE by calculating the dilution factor. The samples of bacteria, fungus and archaea from the 100 pmol libraries with similar barcodes were pooled to form pooled barcoded library in a non-sticky Eppendorf tube, and once again, the pmol concentration was checked. The dilution factor was calculated to get 100 pmol concentration using the formula; Dilution factor = pmol /100. The pooled library was diluted to 100 pmol concentration by adding 10 µL pooled library and calculated amount of Low TE in a non-sticky Eppendorf tube, and from this diluted pooled library 2 µL each was aspirated into 1.5 ml Eppendorf tube to form final pooled library, which was utilized further to prepare template ISPs in Ion One Touch machine. Template positive ion sphere particles were prepared with purified final pooled 100 pmol amplicon library using Ion PGM^{TM} Hi-Q View OT$_2$ Kit in Ion One Touch 2 instrument set up made according to the manufacturer's protocol for 400 base pair read libraries. Sequencing was done using Ion PGM^{TM} sequencer, which used real-time hydrogen ions measurements produced during DNA replication with 850 flows.

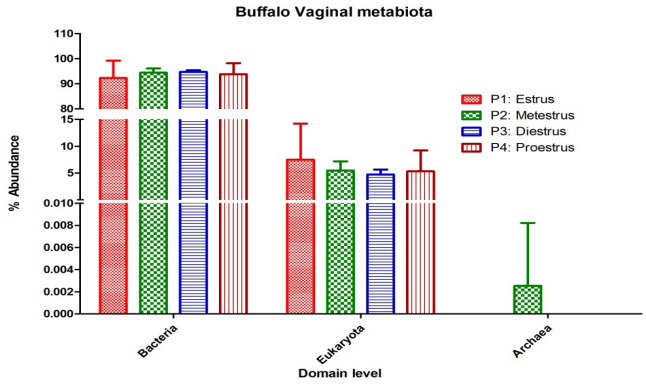

Fig. 1: Domain level standard deviation representing the absolute abundance between four physiological events of buffalo heifers (n=08).

Data Processing and Analysis

The annotation of the data obtained through NGS sequencing of 28 samples vaginal mucus of eight buffaloes (four phase; estrus, proestrus, metestrus and diestrus) were uploaded into the online annotation server MG-RAST. The dynamic trimming option was selected aiming the elimination of low quality sequences, requiring a 15 minimal pherd score of each base and a limit of 5 low quality bases per fragment. The high quality sequences generated were used in subsequent analysis with RNA database. The RNA database chosen were RDP, Greengenes, Silva SSU etc. To increase the accuracy of results in RNA database analysis, the identity was fixed to 98%, e-value 8, and length 80 bp, even though minimum criteria given by the MG-RAST is identity 60%, e-value 5 and length 75 bp. All the results were analyzed on STAMP by one way ANOVA with Tukey's post hoc test with $p < 0.05$. The association between buffalo vaginal microbiota with hormonal parameters E_2 and P_4 was carried out using Pearson's correlation in SPSS® software (SPSS 24, IBM Pvt. Ltd., Bengaluru, India).

Results and Discussion

Libraries Sequencing Data

The rarefaction curve of all 28 sequenced libraries results showed that the samples were satisfactorily represented for all libraries. This data indicate that the environment was adequately explored, giving a true representation of the operational taxonomic unit (OTU).

Vaginal Microbiota and Estrous Cycle

The vaginal microbiota of the buffalo heifers can be grouped into four major domains in which bacteria accounts 93.8%, eukaryotes 5.7%, followed by archaea 0.006% and 0.41 % unclassified sequences. In total 2196 bacterial species, 17 fungal species and two archaeal species were identified. The variability observed among groups in each domain in Fig. 1 and the rhythm of bacteria, archaea, and fungus at a different phylogenetic level during various stages of the estrous cycle is depicted in Table 1.

Bacterial Dynamics during Different Stages of Estrous Cycle

The bacterial domain contained a total of 18 bacterial phyla which can be grouped into high, moderate, and less abundant

Table 1: Dynamics of vaginal microbiota at a different phylogenetic level during four phase of the estrous cycle (Ba: Bacteria; Ar: Archaea; Fu: Fungus)

	Estrus			*Metestrus*			*Diestrus*			*Proestrus*		
	Ba	*Ar*	*Fu*	*Ba*	*Ar*	*Fu*	*Ba*	*Ar*	*Fu*	*Ba*	*Ar*	*Fu*
Phylum	14	0	2	17	1	2	18	0	2	17	0	2
Class	27	0	4	31	1	5	32	0	4	29	0	3
Order	66	0	6	70	1	6	77	0	5	70	0	4
Family	155	0	6	155	1	7	176	0	5	146	0	4
Genus	382	0	8	375	1	9	483	0	7	340	0	4
Species	1154	0	10	1206	2	11	1600	0	8	957	0	4

(>5%, 0.1-5%, and 0.0-0.1%, respectively) groups. The four most highly abundant (>5%) phyla detected were Firmicutes, Actinobacteria, Proteobacteria and Unclassified bacteria. Thermotogae, Bacteriodetes, Fusobacteria, Dienococcus-Thermus, Cyanobacteria, and Teniricutes were six phyla grouped into moderately abundant (0.1-5%) Verrucomicrobia, Synergistetes, Spirochaetes, Chloroflexi, Acidobacteria, Nitrospirae, Chlorobi, Fibrobacteres were grouped into less abundant (0-0.1%) phyla. In earlier studies also, the phyla Firmicutes, Proteobacter, and Bacteroides were observed as abundant groups in buffalo (Mahalingam *et al*., 2019) and Nellore cattle vagina (Laguardia-Nascimento *et al*., 2015). Some high and moderate abundant phyla Fusobacteria, Tenericutes, Firmicutes, Proteobacteria, and Uncultured bacteria were also observed in postpartum buffaloes' normal and endometriotic uterus of postpartum buffaloes (Onnureddy *et al*., 2013), which confirms the migration of vaginal microflora into the uterus and thus playing an important role in fertility. The relative abundance of all 18 bacterial phyla is shown in Fig. 2A. In the present study, unclassified bacteria was one of the abundant group which was also noticed in cattle vagina (Laguardia-Nascimento *et al*., 2015), cattle uterus (Santos *et al*., 2011), and buffalo uterus (Onnureddy *et al*., 2013), suggesting the need for further development of the database. Fusobacteria, which is grouped into a moderately abundant genus in the present study, was also noticed as the dominant phylum during metritis and endometritis in postpartum dairy cows; and Firmicutes, which is abundant phylum in the present study, was the submissive group in endometritis and metritis cows (Santos and Bicalho, 2012). To maintain the reproductive microbial ecosystem, Firmicutes play an important role, or it can serve as an indicator of the health status of the reproductive tract.

Eleven genera Cardiobacterium, Cellulomonas, Amycolatopsis, Pimelobacter, Promicromonospora, Xanthobacter, Rickettsia, Massilia, Desulfonispira, Leptospira and Anerotruncus, out of total 589 bacterial genera detected, were showing significance ($p < 0.05$; Fig. 2B), which belonged to four phyla of the bacterial domain. The species of Pimelobacter detected in the present study was *Pimelobacter simplex*. The culture of *Pimelobacter simplex* VKPM Ac-1632 converts 17 α -Methyltestosterone to Methandrostenolone in the presence of cyclodextrins (Druzhinina *et al*., 2008). The role of Pimelobacter in the vaginal tract of buffalo and its effect on the host hormone needs to be explored further. The species detected in Amycolatopsis genus were *Amycolatopsis sacchari, Amycolatopsis thermoflava, Amycolatopsis sulphurea, Amycolatopsis ultiminotia, Amycolatopsis taiwanensis, Amycolatopsis alba, Amycolatopsis sp. GY034, Amycolatopsis methanolica, Amycolatopsis jejuensis, Amycolatopsis coloradensis* and *Amycolatopsis mediterranei*. Literature suggests that amyclatopsis group is the important source of some antibiotics. *Amycolatopsis mediterranei* produces resistomycin (Wink *et al*., 2003), however, the present study detected its presence but the total hits was zero, hence further studies are needed for confirmation. Cardiobacterium genus has been earlier reported in respiratory and nasal tracts and in the reproductive tract of humans, but for the first time in the buffalo vagina in the present study. *Desulfonispora thiosulfatigenes* strain GKNTAUT has been described as a bacterium able to ferment the organosulfonate taurine (2-aminoethanesulfonate) quantitatively to acetate, ammonia and thiosulfate, which is an unusual metabolic product (Denger *et al*., 1999), and this needs further elucidation.

Eukaryota, Fungus and Archaea Dynamics during Different Stages of Estrous Cycle

Eukaryota contributes about 5.7% of overall vaginal metabiota. Amongst 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 5.3% of total Eukaryota. The abundance of Streptophyta, a plant genus, may be present due to the wallowing habit of buffaloes. Previous study in Nellore cattle has reported less eukaryotes in the vaginal tract (Laguardia-Nascimento *et al*., 2015); however, eukaryotes were abundant in the buffalo vagina in the present study.

The fungal phyla consisted of genus Aspergillus, Penicillium, Cladosporium, Verticillium, Myrothecium, Rhizopus, Kabatiella, Emericella, Peyronellaea, Eurotium,

Candida, and Mycospharella etc. Aspergillus was the abundant genus in the present study, while Mycospharella, an endophytic fungus of soil, was abundant genus in the Nellore cattle vaginal tract (Laguardia-Nascimento *et al*., 2015). The present study results showed the presence of penicilium genus; the species of penicillium detected were *Penicillium purporgenum* and *Penicillium chrysogenum* at 98% identity, while at 60% identity *Penicillium aurantogesium* was noticed. As the buffaloes prefer marshy environment, buffalo vaginal tract may serve as a niche for penicillium group of an organism that needs further validation. All fungal genera with their relative abundance is shown in Fig. 2C.

Archaea was seen during the metestrus stage and its relative abundance was least (0.006%) in overall vaginal microbiome population. However, to the best of knowledge and literature search, this is the first study reporting presence of Archaea during metestrus phase of estrous cycle in buffaloes. The species identified were *Methanobacterium alkaliphilium* and *Methanobacterium Sp. MB4* belonging to the Methanobacterium genus. There was a rise of Archaeal population relative to bacteria in pregnant Nellore cattle (Laguardia-Nascimento *et al*., 2015). Thus, the presence of Archaea during metestrus may be attributed to the influence of hormone P_4. An alkaline environment is favorable for the development of Archaea (Bengtson *et al*., 2012) which is created by P_4 dominance; however, its role in vaginal microbiota needs further elucidation.

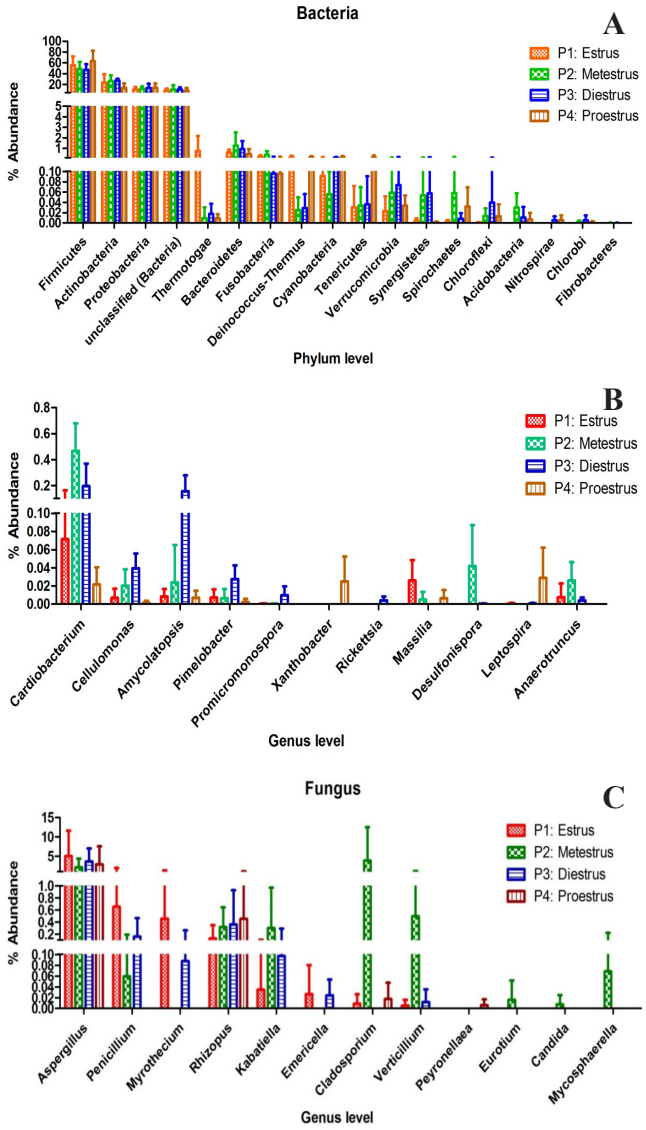

Fig 2: Relative abundance and standard deviation of bacterial phyla (a) and genera (b) and fungal genera (c) during four phase of estrous cycle of eight buffalo heifers

Phase to Phase Fold Change of Vaginal Metabiota during Estrous Cycle

The phase to phase fold change, carried out in Log base 2 value to notice increase or decrease, is shown for estrus (P1/P4), metestrus (P2/P1), diestrus (P3/P2), proestrus (P4/P3), where P1 is estrus, P2 metestrus, P3 diestrus and P4 proestrus in Fig 3 ABC.

At phylum level there is rise of Thermotage both at estrus, diestrus and decrease at metestrus and proestrus. In the metestrus phase, Acidobacteria showed an increase of 11 fold, followed by Chlorobi, Spirochaetes and Fibrobacteres. Verrucomicrobia during luteal phase and decreased in follicular phase. Spirochaetes decreased at diestrus and estrus but increased at proestrus and metestrus. This may be due to fluctuations of hormones at metestrus and proestrus which is in contrast to Thermotoage. Chloroflexi and Chlorobi were increasing during luteal phase and reducing in follicular phase (Fig. 3A).

During estrus to metestrus phase, Desulfonispora genus showed the highest increase of about 12 folds followed by Cardiobacterium and Cellulomonas and Amycolatopsis; while the genus Leptospira, Massilila, Promicromonospora, and Pimelobacter showed a reduction. At diestrus phase, Rickettesia showed a rise of 8 fold which decreased in successive proestrus phase. In metestrus and proestrus, there was a decrease of Pimelobacter and Promicromonospora, which increased at both diestrus and estrus phases. Cellulomonas, Amycolatopsis, Cardiobacterium, Desulfonispora, and Anaeroruncus showed rise at an increase in P_4 and decrease in E_2 (metestrus), but the same group showed a decrease during fall in P_4 and rise in E_2 (proestrus; Fig. 3B).

Myrothrecium, Kabatiella, Emericella and Penicillium genera showed more than nine fold rise during the estrus phase. Aspergillus, Penicillium, Myrithecium, Emericella groups increased during estrus and diestrus but reduced at metestrus and proestrus. Kabatiella and Vitricillium increased with P_4 rise and E_2 fall (metestrus) and decreased with P_4 fall and E_2 rise (proestrus; Fig. 3C). These genera may be having markers for E_2 and P_4 indicating the clue in the identification of host hormone microflora interrelationship.

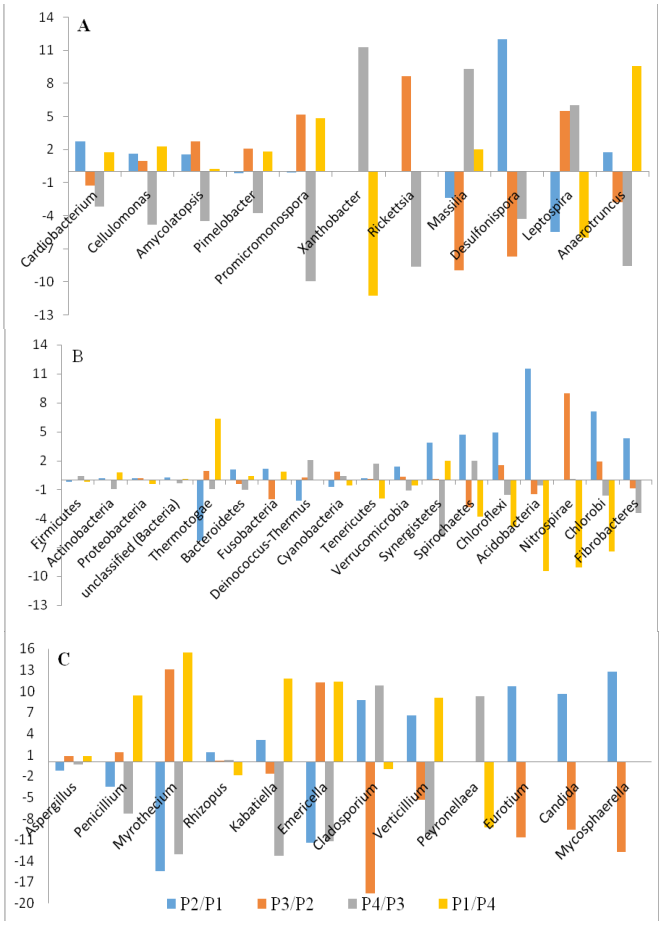

Fig. 3: A. Log_2 indicating phase to phase fold change of bacterial phyla (n=18); **B**. Log_2 indicating phase to phase fold change in bacterial genera ($p < 0.05$), and **C**. Log_2 indicating phase to phase change of fungal genera where P1=Estrus, P2=Metestrus, P3=Diestrus, P4=Proestrus

Table 2: Classification of a bacterial phylum based on abundance during estrous cycles of eight buffalo heifers

Highly abundant phyla (>5%)	*Moderately abundant phyla (0.1-5%)*	*Less abundant phyla (0-0.1%)*
Firmicutes	Thermotogae	Verrucomicrobia
Actinobacteria	Bacteriodetes	Synergistetes
Proteobacteria	Fusobacteria	Spirochaetes
	Dienococcus-thermus	Chloroflexi
	Cyanobacteria	Acidobcteria
Unclassified Bacteria		Nitrospirae
	Teniricutes	Chlorobi
		Fibrobacteres

The phase to phase fold change of all 589 bacterial genera can be grouped into 16 different combinations of high (H) and low (L), wherein the genus showing greater than one fold increase grouped under 'High' and genus showing reduction or no change grouped under 'Low.' Out of 589 genera, 93 showed LHLL, 81 genera LHLH, and 74 genera HLLL (Table 2). This shows that there exists clear dynamics in vaginal microflora at genus level.

Association of Vaginal Metabiota with Estradiol and Progesterone

The values of E_2 and P_4 hormones during estrus, metestrus, diestrus and proestrus of synchronized estrous cycle were 3.98±0.81 pg/ml and 0.46±0.008 ng/ml, 3.3±0.62 pg/ml and 1.8±0.34 ng/ml, 2.47±0.7 pg/ml and 4.19±0.57 ng/ml and 2.86±0.56 pg/ml and 0.51±0.07 ng/ml, respectively. The mean E_2 and P_4 values differed between four periods of the estrous cycle ($p < 0.05$). The hormonal trend during the four periods of the estrous cycle was within the normal range as reported earlier (Mondal *et al*., 2007, 2010). Proteobacteria one highly abundant phylum, and Nitrospirae, one less abundant phylum, demonstrated a negative correlation ($p < 0.05$) with E_2. Out of 589 bacterial genera, 48 genera showed a positive correlation, and four genera showed a negative correlation with plasma P_4 ($p < 0.05$; Table 3). Genera Helicobacter, Thermodesulfovibrio, Thermoflavimicrobium and Agarivorans showed a negative correlation, while genera Microcystis, Haella, Alicyclobacillus, Aggregatibacter and Citrobacter showed a positive correlation with E_2 ($p < 0.05$). Pearson's correlation results of 12 genera of fungal and two archea did not reveal any association with E_2 and P_4 during different stages of estrous cycle. The results suggest that reproductive hormones E_2 and P_4 have a clear influence on the abundance and rhythmicity of microbiota during the estrous cycle.

Conclusions

Our study demonstrated a total of 2196 bacterial species, 17 fungal species, and two archaeal species, which confirm that the buffalo vagina has rich microflora diversity. The bacterial domain contained a total of 18 bacterial phyla, highly abundant phyla Firmicutes, Actinobacteria, Proteobacteria and Unclassified bacteria, and moderate abundant Thermotogae, Bacteriodetes, Fusobacteria, Dienococcus-Thermus, Cyanobacteria, and Tenericutes. Out of total 589 bacterial genera detected, Amycolatopsis, Cardiobacterium, Cellulomonas, Pimelobacter, Promicromonospora, Xanthobacter, Rickettsia Massilia, Desulfonispira, Leptospira, and Anerotruncus showed the statistical significance of bacterial domain, and two fungal phyla detected were Ascomycota and Unclassified Fungi. Estradiol showed a negative correlation with Proteobacteria, a highly abundant and Nitrospirae less abundant phyla. Helicobacter, Thermodesulfovibrio, Thermoflavimicrobium, and Agarivorans showed a negative correlation with E_2, while Microcystis, Haella, Alicyclobacillus, Aggregatibacter and Citrobacter showed a positive correlation. Forty-eight genera showed positive correlation and four genera showed negative correlation with plasma P_4. Indeed the study

Table 3: Pearson's correlation of bacterial genera with blood progesterone (P_4) and estradiol (E_2) levels of buffalo heifers

Genus	*P4*	*P value*	*Genus*	*P4*	*P value*	*Genus*	*P4*	*P value*	*Genus*	*E2*	*P value*
Bulleidia	0.969*	0.031	Syntrophomonas	0.980*	0.02	Blastobacter	0.981*	0.019	Helicobacter	-0.982*	0.018
Pseudobutyrivibrio	0.972*	0.028	Salinibacterium	1.000**	0	Soehngenia	0.973*	0.027	Thermodesulfovibrio	-0.969*	0.031
Sporolactobacillus	0.953*	0.047	Sphaerobacter	0.954*	0.046	Eggerthella	0.952*	0.048	Thermoflavimicrobium	-0.968*	0.032
Catenibacterium	0.969*	0.031	Unclassified (Peptococcaceae)	0.998**	0.002	Catenuloplanes	0.995**	0.005	Agarivorans	-0.961*	0.039
Jonesia	0.967*	0.033	Unclassified (Epsilonproteobacteria)	0.997**	0.003	Mycobacterium	0.977*	0.023	Microcystis	0.958*	0.042
Geodermatophilus	0.970*	0.03	Megamonas	0.962*	0.038	Megasphaera	0.996**	0.004	Hahella	0.954*	0.046
Phormidium	0.964*	0.036	Faecalibacterium	0.955*	0.045	Actinoallomurus	0.992**	0.008	Alicyclobacillus	0.973*	0.027
Beutenbergia	0.956*	0.044	Coprococcus	0.995**	0.005	Halomonas	0.962*	0.038	Aggregatibacter	0.981*	0.019
Aeromicrobium	0.956*	0.044	Virgibacillus	0.964*	0.036	Aphanizomenon	0.974*	0.026			
Cellulomonas	0.978*	0.022	Myroides	0.993**	0.007	Unclassified (Rhodobacteraceae)	0.980*	0.02			
Amycolatopsis	0.978*	0.022	Alkalibacterium	0.963*	0.037	Serinicoccus	0.966*	0.034			
Pimelobacter	0.953*	0.047	Thermoactinomyces	0.993**	0.007	Peredibacter	0.954*	0.046			
Promicromonospora	0.953*	0.047	Marinococcus	0.984*	0.016	Cryptobacterium	0.970*	0.03			
Acetivibrio	0.973*	0.027	Oerskovia	0.954*	0.035	Agrobacterium	-0.977*	0.023	Citrobacter	0.991**	0.009
Renibacterium	0.957*	0.043	Halothermothrix	0.969*	0.046	Sphingomonas	-0.981*	0.019			
Bartonella	0.988*	0.012	Segniliparus	0.974*	0.031	Nocardiopsis	-0.964*	0.036			
Unclassified (Clostridiales)	0.983*	0.017	Exiguobacterium	0.996**	0.026	Delftia	-0.965*	0.004			
Unclassified (Erysipelotrichaceae)	0.969*	0.031									

** $p < 0.01$, * $p < 0.05$

demonstrated rich diversity and dynamics of the microbiota of the vagina in buffalo heifers. At different phylogenetic level, the microbiota of the vagina revealed an association with E_2 and P_4 hormones during an estrous cycle. The present study demonstrated novel findings and is the first of its kind in buffalo heifers; however, further research is warranted for validation.

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