

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 NAAS Rating (2026): 5.29
 IJABR 2026; 10(6): 477-481
www.biochemjournal.com
 Received: 01-04-2026
 Accepted: 06-05-2026

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Metagenomic analysis of the vaginal microbiome in cycling buffaloes (*Bubalus bubalis*) through 16S rRNA gene sequencing

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i6f.8664>

Abstract

The vaginal microbiome plays an important role in maintaining reproductive health and fertility by regulating microbial homeostasis within the reproductive tract. The present study was undertaken to characterize and compare the vaginal microbiome of cycling buffaloes during estrus and diestrus using 16S rRNA gene sequencing. Vaginal samples were collected from 10 healthy cycling buffaloes during estrus and diestrus and subjected to metagenomic analysis to determine the composition and diversity of bacterial communities. The results revealed that the vaginal microbiome was dominated by members of the phyla Bacillota, Bacteroidota, Pseudomonadota, and Actinomycetota in both reproductive stages. Bacillota was the predominant phylum in estrus and diestrus buffaloes. Estrus samples exhibited comparatively higher abundances of Bacillota and Bacteroidota, whereas diestrus samples showed relatively greater proportions of Pseudomonadota and Actinomycetota. At the genus level, *Leuconostoc* sp. was abundant at estrus when compared to *Segatella copri* during diestrus. Several bacterial taxa were shared between estrus and diestrus, indicating the presence of a common core microbiota. However, variations in the abundance of specific bacterial populations suggested that reproductive stage influences microbial community structure. The findings demonstrate that the vaginal microbiome of buffaloes is dynamic and undergoes stage-dependent shifts during the estrous cycle. This study provides baseline information on the normal vaginal microbiota of healthy buffaloes and contributes to a better understanding of host-microbe interactions within the reproductive tract.

Keywords: Buffalo, vaginal microbiome, estrus, diestrus

Introduction

The advent of culture-independent molecular techniques, particularly 16S rRNA gene sequencing, has greatly expanded our understanding of the reproductive microbiome. Studies in cattle have demonstrated that the vaginal microbiota is predominantly composed of members of the phyla Bacillota (Firmicutes), Bacteroidota (Bacteroidetes), Pseudomonadota (Proteobacteria), and Actinomycetota (Actinobacteria), although their relative abundance varies among breeds, physiological states, and reproductive stages (Swarz *et al.*, 2014; Laguardia-Nascimento *et al.*, 2015; Nesengani *et al.*, 2017) [8, 2, 4]. Similarly, Clemmons *et al.* (2017) [1] reported that the vaginal microbiota of dairy cows exhibits high diversity and plays an important role in maintaining reproductive tract health.

Sex steroid hormones are recognized as major determinants of microbial composition within the reproductive tract. Fluctuations in estrogen and progesterone concentrations during the estrous cycle alter the vaginal environment, including pH, mucus secretion, epithelial turnover, and local immune responses, thereby influencing microbial colonization and community structure (Neuman *et al.*, 2015; Wessels *et al.*, 2018) [5, 9]. Studies in buffaloes have demonstrated distinct changes in vaginal bacterial populations across different phases of the estrous cycle, suggesting a close interaction between host endocrine status and microbial ecology (Mahalingam *et al.*, 2019; Patel, 2019) [3, 6].

Although reproductive microbiome research has advanced considerably in cattle, information regarding the vaginal microbiota of buffaloes remains limited. Furthermore, the microbial dynamics associated with estrus and diestrus in cycling buffaloes has not been fully elucidated.

A comprehensive understanding of these microbial communities may provide valuable insights into reproductive physiology and contribute to the development of microbiome-based strategies for improving reproductive efficiency. Therefore, the present study was undertaken to characterize and compare the vaginal microbiome of buffaloes during estrus and diestrus using 16S rRNA gene sequencing.

Materials and Methods

The study was conducted using ten healthy and cycling she buffaloes (2-3 parity, BCS 3-4) presented to veterinary dispensaries of Yadgir district (Karnataka state, India). All the animals were apparently healthy and didn't receive any antibiotic treatment during 1 month prior to sampling. Selected animals were negative for the subclinical endometritis by white side test. Vaginal samples were collected aseptically from each animal during estrus and during diestrus (10-12 days post-estrus). The swabs were stored in phosphate-buffered saline (PBS) and transferred to the laboratory in cold chain. The samples were stored at -80 °C until further processing.

For metagenomic analysis, DNA was extracted from vaginal swab samples using the Xploreagen Microbial DNA Extraction Kit. Amplification of the bacterial 16S rRNA gene was performed using universal primers targeting conserved regions. The forward primer (16sF-5' CCTACGGGNGGCWGCAG 3') and reverse primer (16sR-5' GACTACHVGGGTATCTAATCC 3') enabled amplification of the target region, and the resulting amplicons were subsequently used for sequencing-based microbiome analysis. PCR amplification was carried out using 40 ng of genomic DNA along with 10 pM of each primer.

Taxonomic classification was performed using the NCBI database, with particular focus on the V3-V4 hypervariable region of the 16S rRNA gene. Sequencing output generated in binary base call (BCL) format was converted into FASTQ files. Representative sequences were aligned against the NCBI 16S rRNA database to assign taxonomic identities.

This resulted in the generation of an OTU table containing taxa and their relative abundances across samples. The abundance profiles, along with dominant microbial taxa, were compiled and visualized using Microsoft Excel.

The approvals of the Institutional Animal Ethics Committee (IAEC) for the present study was obtained from Veterinary College Hebbal, Bangalore (KVAFSU University) vide IAEC No. VCH/IAEC/2025/25.

Results

In the estrus group, vaginal microbiota of buffaloes constituted of Bacillota 47.33% of the total relative abundance, followed by Bacteroidota (22.61%), Pseudomonadota (15.87%), and Actinomycetota (9.92%). Minor phyla included Verrucomicrobiota (1.58%) and Thermotogota (1.11%), while other phyla such as Mycoplasmatota, Spirochaetota, Campylobacterota, Chloroflexota, Deferribacterota, Fusobacteriota, Gemmatimonadota, Synergistota, and Thermodesulfobacteriota were present in relatively low abundances (<1%). In contrast, the diestrus group exhibited a relatively lower abundance of Bacillota (38.24%), while Pseudomonadota (22.12%) and Actinomycetota (15.15%) were comparatively higher than in the estrus group. Bacteroidota accounted for 18.90%, followed by Verrucomicrobiota (2.70%) and Thermotogota (0.74%) (Table 1). Additionally, several low-abundance phyla, including Campylobacterota, Thermodesulfobacteriota, Mycoplasmatota, Fusobacteriota, Spirochaetota, Acidobacteriota, Myxococcota, Planctomycetota, Gemmatimonadota, Nitrospirota, Bdellovibrionota, Chlamydiota, Chloroflexota, Deferribacterota, Deinococcota, and Synergistota, were detected in the diestrus group. Overall, while both groups shared a similar core microbiota, notable differences in relative abundance were observed, with estrus vaginal samples showing higher representation of Bacillota and Bacteroidota, whereas diestrus samples exhibited increased abundance of Pseudomonadota and Actinomycetota (Figure 1).

Table 1: Top 10 phyla identified in the vagina and their relative abundance in cycling buffaloes during natural estrus and natural diestrus

Phylum	Relative abundance (%)	
	Natural estrus	Natural diestrus
Bacillota	47.33	38.24
Bacteroidota	22.61	18.90
Pseudomonadota	15.87	22.12
Actinomycetota	9.92	15.15
Verrucomicrobiota	1.58	2.70
Thermotogota	1.11	0.74
Mycoplasmatota	0.55	0.19
Spirochaetota	0.27	*
Campylobacterota	0.25	0.66
Chloroflexota	0.08	*
Thermodesulfobacteriota	*	0.42
Fusobacteriota	*	0.14

Note: * indicates abundance has not depicted against some phylum as they are absent in that specific stage of cycle

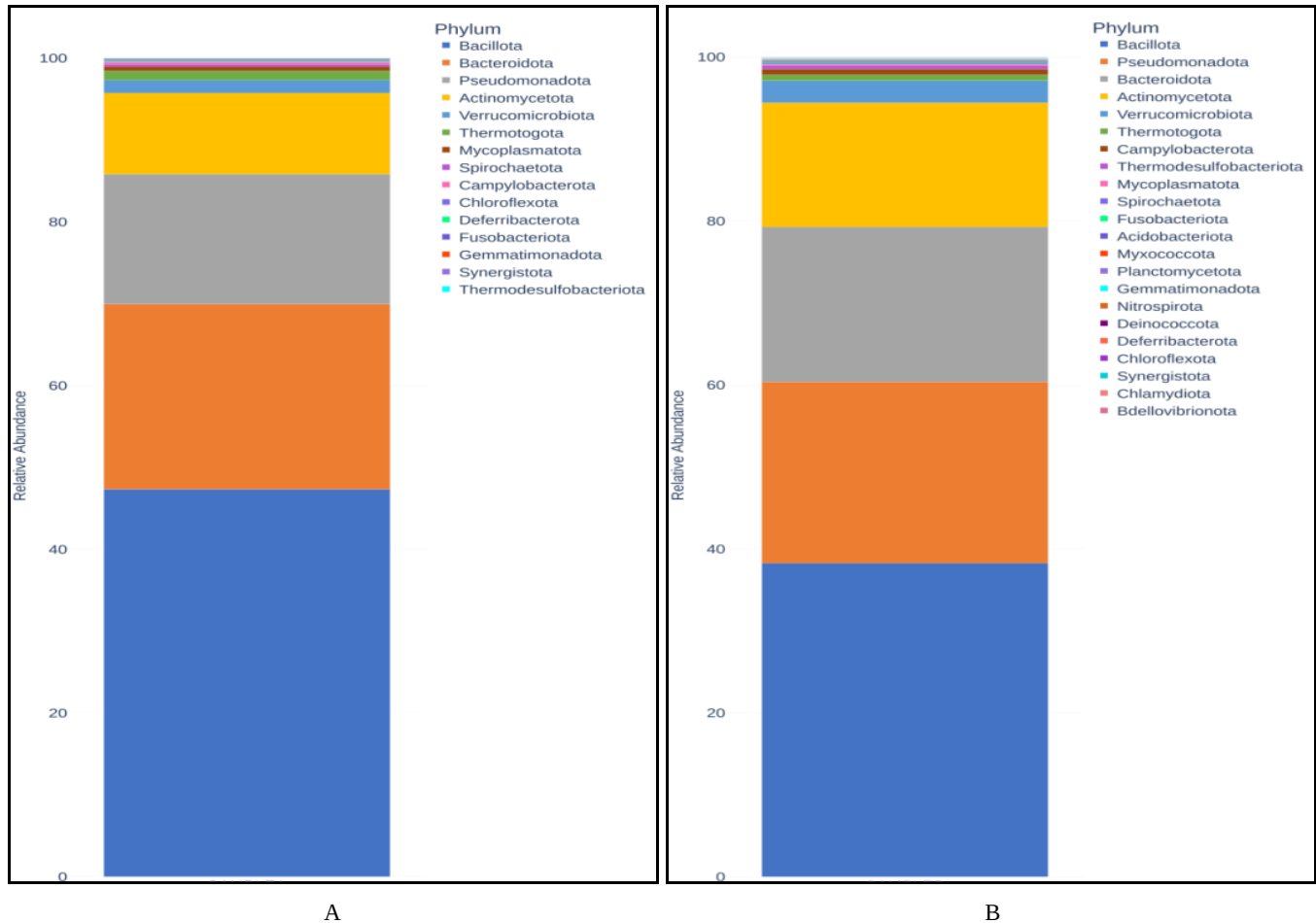


Fig 1: Stacked bar diagram of phylum level vaginal microbiota after 16S rRNA sequencing in natural estrus (A) and natural diestrus (B) buffaloes

At the species level, the vaginal microbiota of estrus group, the microbial community was predominantly dominated by *Leuconostoc* sp. (12.48%), followed by *Segatella copri* (9.04%), *Leyella stercorea* (5.34%), and *Caulobacter* sp. (5.02%). Other notable species included *Staphylococcus* sp. (4.37%), *Succinivibrio dextrinosolvens* (3.91%), *Bifidobacterium* sp. (2.90%), and *Collinsella aerofaciens* (2.59%). Several additional taxa such as *Lactiplantibacillus* sp., *Leuconostoc lactis*, *Ileibacterium valens*, and *Faecalibacterium* sp. were present in moderate abundance (2-3%). In contrast, the diestrus group exhibited a different pattern of dominance, with *Segatella copri* (7.56%) being the most abundant species, followed by *Corynebacterium* sp. (6.98%), *Lactococcus* sp. (6.56%), and *Psychrobacter* sp. (4.22%). Other prominent species included *Leyella stercorea* (3.77%), *Lactiplantibacillus* sp. (3.48%), *Caulobacter* sp. (3.29%), and *Stenotrophomonas* sp. (3.13%) (Table 2).

Comparative analysis revealed that *Leuconostoc* sp. and *Bifidobacterium* sp. were more abundant in the estrus group, whereas *Corynebacterium* sp., *Lactococcus* sp., and *Psychrobacter* sp. were relatively enriched in the diestrus group. Notably, *Segatella copri* was identified as a common dominant species in both groups, although its relative abundance differed between estrus and diestrus samples. A large number of species were detected at low relative abundance (<1%) in both groups, indicating a highly diverse microbial community with a substantial proportion of rare taxa (Figure 2).

Table 2: Top 20 bacterial species identified in the vagina and their relative abundance in cycling buffaloes during natural estrus and natural diestrus

Species	Relative abundance (%)	
	Natural estrus	Natural diestrus
<i>Leuconostoc</i> sp.	12.48	2.51
<i>Segatella copri</i>	9.04	7.56
<i>Leyella stercorea</i>	5.34	3.77
<i>Caulobacter</i> sp.	5.02	3.29
<i>Staphylococcus</i> sp.	4.37	2.27
<i>Succinivibrio dextrinosolvens</i>	3.91	1.23
<i>Bifidobacterium</i> sp.	2.90	1.75
<i>Collinsella aerofaciens</i>	2.59	1.09
<i>Lactiplantibacillus</i> sp.	2.37	3.48
<i>Leuconostoc lactis</i>	2.25	*
<i>Ileibacterium valens</i>	2.21	1.94
<i>Faecalibacterium</i> sp.	2.16	1.06
<i>Segatella hominis</i>	1.85	*
<i>Akkermansia muciniphila</i>	1.58	2.60
<i>Lactococcus</i> sp.	1.43	6.56
<i>Lactobacillus</i> sp.	1.32	1.47
<i>Ligilactobacillus</i> sp.	1.30	2.79
<i>Prevotellamassilia timonensis</i>	1.22	*
<i>Bacteroides fragilis</i>	1.18	*
<i>Mesotoga infera</i>	1.11	*
<i>Corynebacterium</i> sp.	*	6.98
<i>Psychrobacter</i> sp.	*	4.22
<i>Stenotrophomonas</i> sp.	*	3.13
<i>Serratia</i> sp.	*	2.41
<i>Walteria intestinalis</i>	*	0.87

Note: * indicates abundance has not depicted against some species as they are absent in that specific stage of cycle

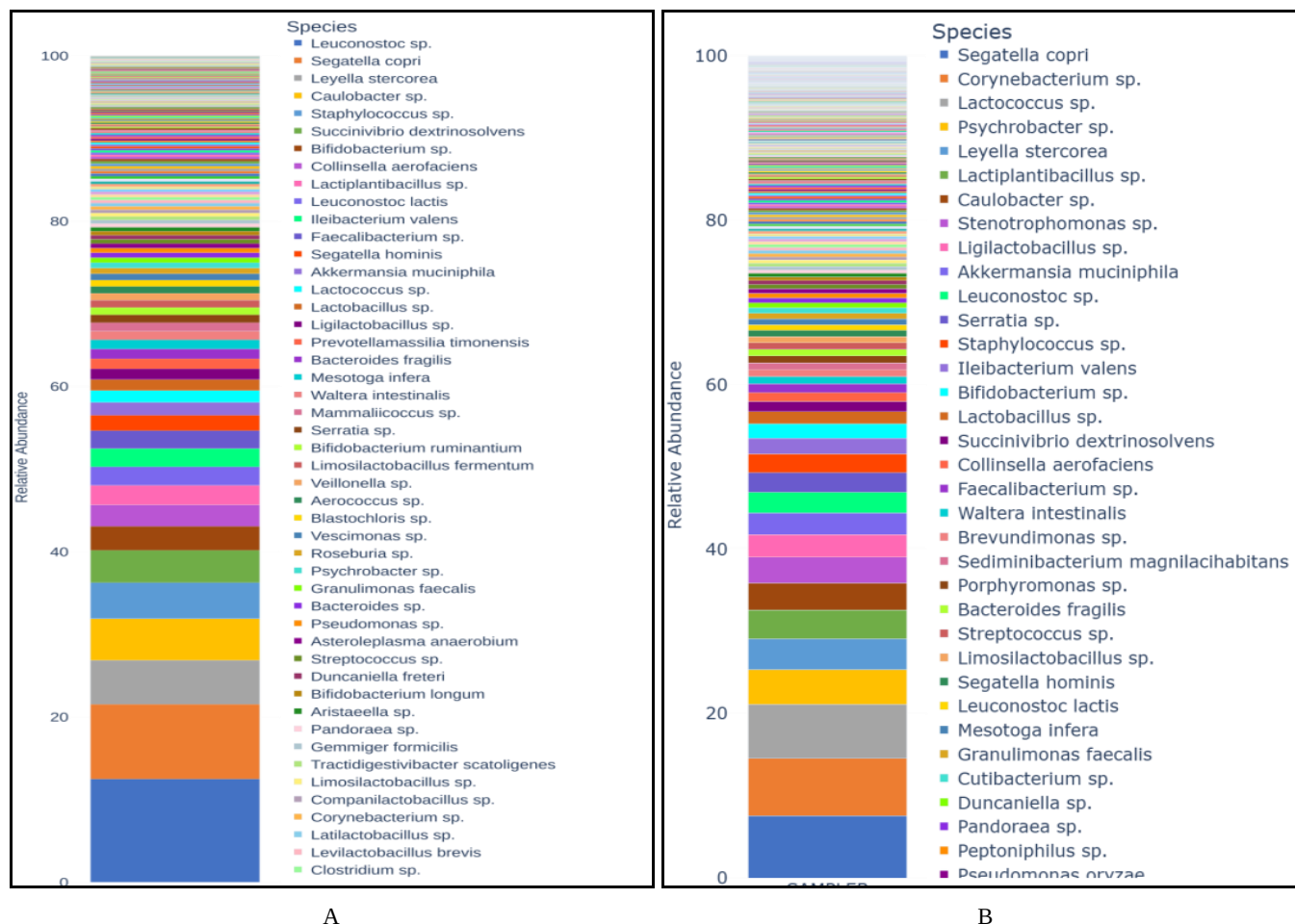


Fig 2: Stacked bar diagram of species level vaginal microbiota after 16S rRNA sequencing in natural estrus (A) and natural diestrus (B) buffaloes

Discussion

The present study revealed that Bacillota (Firmicutes) was the predominant phylum in both estrus (47.33%) and diestrus (38.24%) buffaloes, followed by Bacteroidota, Pseudomonadota (Proteobacteria), and Actinomycetota. However, a comparatively higher abundance of Bacillota and Bacteroidota was observed during estrus, whereas diestrus samples exhibited increased proportions of Pseudomonadota and Actinomycetota. These findings indicate that although a common core microbiota was maintained across the estrous cycle, fluctuations in the relative abundance of major bacterial phyla occurred between reproductive stages.

The predominance of Bacillota (Firmicutes) observed in the present study is in agreement with previous reports in cattle and buffaloes. Laguardia-Nascimento *et al.* (2015) [2] reported 40-50% of the vaginal microbiota in Nellore cattle; while Nesengani *et al.* (2017) [4] recorded 51.7-58.8% in healthy cattle; also 65.9% was observed in postpartum dairy cows (Clemmons *et al.*, 2017) [1]. In buffaloes, Patel (2018) [7] reported 32.50% in estrus and 55.32% in diestrus. The high abundance of Firmicutes may be attributed to their ability to colonize mucosal surfaces and contribute to the maintenance of reproductive tract homeostasis through competitive exclusion of opportunistic pathogens. Members of this phylum are known to participate in carbohydrate metabolism and mucosal nutrient turnover, which may support the dynamic physiological changes occurring during estrus. The elevated abundance during estrus may therefore reflect hormonal influences and alterations in the vaginal

microenvironment associated with increased estrogen concentrations.

In contrast, Pseudomonadota and Actinomycetota were comparatively more abundant during diestrus. Similar observations have been reported by Mahalingam *et al.* (2019) [3], who demonstrated marked variations in the relative abundance of Proteobacteria and Actinobacteria across different stages of the buffalo estrous cycle. Patel (2019) [6] also reported Proteobacteria as the predominant phylum during both estrus and diestrus, accompanied by substantial representation of Actinobacteria. The increased abundance of these phyla during diestrus may be associated with progesterone-mediated changes in vaginal secretions, pH, and nutrient availability, which can selectively favor the proliferation of specific bacterial populations.

The detection of low-abundance phyla including Verrucomicrobiota, Mycoplasmatota, Spirochaetota, Campylobacterota, Fusobacteriota, Synergistota, and Thermodesulfobacteriota in both groups further highlights the complexity of the buffalo vaginal ecosystem. Similar minor phyla have also been reported in buffaloes and cattle (Patel, 2018; Patel, 2019 and Mahalingam *et al.*, 2019) [7, 6, 3]. Although present in small proportions, these microbial populations may contribute to ecological stability and functional diversity within the reproductive tract.

The findings of the present study indicate that the vaginal microbiota of buffaloes is primarily dominated by Bacillota, Bacteroidota, Pseudomonadota, and Actinomycetota. The observed shifts in relative abundance between estrus and diestrus suggest that cyclical hormonal changes influence

the composition of the vaginal microbial community, thereby contributing to the dynamic microbial ecology of the buffalo reproductive tract.

At the species level, *Leuconostoc* sp. was the most abundant species during estrus, whereas *Segatella copri* predominated during diestrus. The increased abundance of *Bifidobacterium* sp. during estrus further supports the notion that estrogen-associated conditions favor beneficial bacterial populations. Conversely, *Corynebacterium* sp., *Lactococcus* sp., *Psychrobacter* sp., and *Stenotrophomonas* sp. were relatively enriched during diestrus, indicating a shift in microbial composition associated with changes in reproductive physiology. Similar phase-dependent alterations in bacterial populations have previously been reported in buffaloes and cattle, suggesting that the estrous cycle exerts a significant influence on vaginal microbial ecology (Mahalingam *et al.*, 2019; Patel, 2019) [3, 6].

The findings of the present study demonstrate that although estrus and diestrus buffaloes share a common core microbiota, the relative abundance of several dominant genera and species varies considerably between reproductive stages. These variations are likely driven by cyclical hormonal changes, alterations in vaginal secretions, epithelial turnover, and immune modulation, all of which contribute to shaping the vaginal microbial ecosystem throughout the estrous cycle. The predominance of beneficial and commensal bacterial populations during both reproductive stages indicates the presence of a stable microbial community that may contribute to maintaining reproductive tract health and microbial homeostasis.

Conclusion

The present study successfully characterized the vaginal microbiome of cycling buffaloes during estrus and diestrus using 16S rRNA gene sequencing and demonstrated distinct variations in microbial composition between the two reproductive stages.

Furthermore, the identification of specific bacterial taxa associated with estrus and diestrus provides valuable baseline information on the normal vaginal microbiota of healthy buffaloes. Such information may serve as a reference for future investigations aimed at understanding microbial alterations associated with reproductive disorders, infertility, and postpartum anestrus.

Acknowledgement

The authors express their gratitude to the Department of Animal Husbandry and Veterinary Services, Yadgir District, Government of Karnataka for providing manual support and helping hands during the sample collections at dispensaries level.

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