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Metagenome Analysis of Major Carbohydrate-utilising Rumen Bacteria in Lactating Cows with Sub-acute Rumen Acidosis

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ABSTRACT

The study was conducted during the months of December, 2024 to September, 2025 at the College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India, with the objective of evaluating the changes in major carbohydrate-utilising rumen bacterial population and their diversity through metagenomic analysis in animals affected with subacute rumen acidosis (SARA) before and after probiotic supplementation. In the present study, rumen microbial communities in healthy, SARA-affected, and probiotic-supplemented SARA cattle were characterised using 16S rRNA (V3-V4) amplicon sequencing of rumen liquor samples collected from lactating crossbred cows. Metagenomic analysis revealed that *Bacteroidetes* particularly members of the genus *Prevotella,* and Firmicutes predominated, followed by *Proteobacteria* and *Fibrobacteres*. SARA was associated with lowered rumen pH, reduced microbial alpha diversity, depletion of fibrolytic *Firmicutes* including members of *Clostridia*, *Lachnospiraceae*, *Oscillospiraceae*, along with the enrichment of acid-tolerant *Proteobacteria* such as *Succinivibrio* and *Ruminobacter*, reflecting a dysbiotic microbial community in the rumen. Despite these changes, *Bacteroidetes* (*Prevotella*) continued to remain predominant. Probiotic supplementation partially restored rumen pH, improved microbial richness and diversity, reduced *Proteobacteria* and increased fibrolytic taxa, including *Bacteroidetes* (*Prevotella*) and *Firmicutes*. Furthermore, beta diversity showed distinct clustering among the healthy, SARA, and probiotic supplemented groups, confirming treatment-specific microbial profiles. Overall, these findings indicated that SARA induced major microbial alterations, whereas probiotic supplementation supported the partial restoration of rumen microbial balance and stability.

KEYWORDS: Sub-acute rumen acidosis, metagenome, abundance, diversity, probiotics

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1. INTRODUCTION

The rumen microbiome represents a dynamic ecosystem that is fundamental to the digestive physiology and overall metabolic health of ruminants. This complex community is responsible for the degradation of plant-derived carbohydrates, proteins, and lipids, ultimately converting them into volatile fatty acids (VFAs), which serve as the primary energy source for the host (Bath et al., 2013; Firkins and Yu, 2015). Beyond its role in fermentation, the microbiome is indispensable for the synthesis of essential nutrients, such as amino acids and vitamins, while simultaneously supporting immune function by maintaining the structural integrity of the rumen epithelium (Oetzel, 2017; Liu et al., 2021). This phylogenetically diverse consortium is composed of anaerobic bacteria, fungi, methanogenic archaea, ciliate protozoa, and viruses (O'Hara et al., 2020). Quantitatively, bacteria account for approximately half of the microbial genetic material, followed closely by protozoa in biomass, while fungi represent roughly 2% and methanogenic archaea comprise between 2% and 4% of the total microbial abundance (Nagaraja, 2016). The maintenance of rumen homeostasis depends entirely on the balanced and synergistic interactions among these microbes (Cruz et al., 2016). However, dietary imbalances, specifically the overconsumption of rapidly fermentable concentrates, can disrupt this delicate equilibrium and precipitate sub-acute rumen acidosis (SARA). This condition is characterized by a prolonged reduction in ruminal pH, typically falling within the range of 5.2 to 5.8, due to the rapid accumulation of organic acids (Abdela, 2016; Srivastava et al., 2021). Under the acidic conditions of SARA, the microbial landscape undergoes a significant shift; there is a notable suppression of essential fiber-degrading bacteria (Zhang et al., 2022), while acid-tolerant species begin to flourish and dominate the environment (Petri et al., 2017; Zhu et al., 2018; Fan et al., 2024; Castillo-Lopez et al., 2025). This dysbiosis impairs nutrient utilization, reduces overall productivity, and compromises animal health, a phenomenon commonly observed in high-producing dairy herds during early and mid-lactation. The reduction in beneficial cellulose and starch-degrading bacteria directly decreases energy extraction and performance, while the proliferation of potentially harmful bacteria may promote local inflammation and heighten the risk of systemic disorders. To address the challenges posed by SARA, probiotics have been proposed as a robust therapeutic strategy. These microbial supplements aim to restore homeostatic balance by stabilizing rumen pH, suppressing lactic acid-producing pathogens, and enhancing beneficial microbial populations. By optimizing the ruminal environment, probiotics enhance the efficiency of fiber digestion and mitigate the systemic inflammation associated with dysbiosis (Khan et al., 2016; Arowolo and He, 2018; Tun et al., 2020; Amin and Mao, 2021). To accurately quantify these shifts, researchers increasingly rely on culture-independent techniques like 16S ribosomal RNA (rRNA) gene sequencing. This method identifies complex microbial consortia by targeting a universal marker gene present in all bacteria and archaea. The 16S rRNA gene, spanning approximately 1,550 base pairs, functions as a reliable "molecular clock." It contains nine hypervariable regions (V1-V9) flanked by conserved sequences, allowing for precise taxonomic classification and the estimation of community diversity through comparison with global reference databases (Duchene et al., 2016; Weinroth et al., 2022). Therefore, the present study was undertaken to evaluate the changes in major carbohydrate-utilizing rumen bacterial populations. By utilizing high-throughput metagenome analysis, we aim to characterize the structural and functional shifts in microbial diversity within SARA-affected animals both before and after probiotic supplementation, providing deeper insights into the recovery of the ruminal ecosystem.

2. MATERIALS AND METHODS

2.1. Experimental design

The study was performed over the period from December, 2024 to September, 2025 at College of Veterinary and Animal Sciences, Mannuthy, Thrissur, and was conducted to compare the diversity and relative abundance of rumen microbial communities in healthy, SARA-affected, and probiotic-supplemented cattle using 16S rRNA metagenomic analysis. Eighteen lactating crossbred cows maintained under uniform feeding and management conditions at the University Livestock Farm under Kerala Veterinary and Animal Sciences University, Mannuthy, were included. Animals were divided into three groups (n=6 per group): (i) apparently healthy cattle (control or RL), (ii) SARA-affected cattle (SARA, rumen pH≤5.8), and (iii) SARA-affected cattle after two weeks of probiotic supplementation (PRO). The probiotic bolus contained *Saccharomyces cerevisiae* (10^9 CFU bolus^{-1}), *Lactobacillus sporogenes* (10^6 CFU bolus^{-1}), and *Aspergillus oryzae* (10^6 CFU bolus^{-1}), and was administered daily for 14 days for the SARA-affected animals.

2.2. Sample collection and DNA extraction

Rumen liquor was collected using a flexible stomach tube connected to a vacuum pump, targeting the ventral sac of the rumen. The first 200 ml were discarded to avoid saliva contamination. Samples were filtered through sterile muslin cloth and stored at −80°C until analysis. Rumen pH was measured immediately after collection.

The genomic DNA was extracted using the QIAamp Fast

DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. DNA quality and concentration were assessed using a NanoDrop™ 2000C spectrophotometer.

2.3. Metagenomic profiling and analysis

Equal volumes of DNA from six animals per group (control, SARA-affected and probiotic-supplemented) were pooled and subjected to 16S rRNA (V3-V4) amplicon sequencing. Libraries were prepared using the Nextera XT Index Kit (Illumina), validated on an Agilent 4200 TapeStation and sequenced on the Illumina MiSeq platform (C-Six Labs Pvt. Ltd.). Reads were quality-checked (FastQC), trimmed (Q<30; TrimGalore v0.6.10) and merged (Fastq-join). Taxonomic classification was performed against the NCBI RefSeq 16S rRNA database using Centrifuge and visualised with Pavian and Krona Tools (v2.8.1). Alpha- and beta-diversity analyses were conducted in R (vegan), and taxonomic distributions were presented as Krona charts and comparative plots.

3. RESULTS AND DISCUSSION

3.1. Sample collection and DNA extraction

Rumen liquor (300 ml) was collected from six healthy and six SARA-affected cows at University Livestock Farm under Kerala Veterinary and Animal Sciences University, Mannuthy, before and after probiotic supplementation. Samples were obtained using a stomach tube and filtered. The pH was measured immediately (Table 1), followed by storage at −80°C. Animals with rumen pH≤5.8 (range 5.6–5.8) were classified as SARA-affected according to Li et al. (2013). In agreement with Pinloche et al. (2013) and Goto et al. (2016), probiotic supplementation in the present study significantly increased rumen pH (p=0.001; t=6.508) from 5.75±0.02 to 6.91±0.16, indicating improved ruminal buffering and stabilization of fermentation.

Table 1: The pH of the rumen liquor samples collected from healthy, SARA-affected and probiotics-supplemented cows

Healthy		SARA		Probiotic supplemented	
Animal No.	pH	Animal No.	pH	Animal No.	pH
E-492	7.5	E-176	5.8	E-176	6.79
E-451	9.18	E-506	5.71	E-506	7.56
E-104	7.25	E-226	5.75	E-226	6.45
E-491	6.9	E-446	5.79	E-446	6.7
E-417	7.84	E-396	5.66	E-396	7.2
E-439	8.27	E-422	5.8	E-422	6.8

Genomic DNA was extracted and measured using a NanoDrop spectrophotometer; all samples showed A260/A280 ratios of 1.8-2.0, indicating good purity

3.2. Metagenomic profiling

Metagenomic profiling targeting the V3-V4 regions of the 16S rRNA gene was performed to characterize the bacterial communities in rumen liquor from healthy, SARA-affected, and probiotic-supplemented cows. The 16S rRNA gene is a widely accepted universal marker for prokaryotic community analysis (de Souza et al., 2025). Sequencing yielded high-quality reads with satisfactory base-call accuracy. The rarefaction curves (Figure 1) confirmed adequate sequencing depth across control, SARA, and probiotic groups, indicating sufficient sampling coverage and comprehensive representation of microbial communities in all treatments.

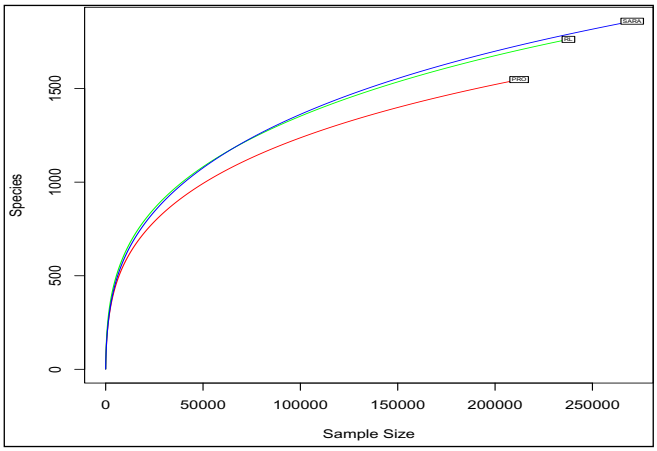

Figure 1: Rarefaction curves of species for experimental control (green), SARA (blue) and PRO (red) groups

3.2.1. Relative abundance

3.2.1.1. Phylum level

Domain-level analysis showed that bacteria (~99%) predominated, with archaea <0.1%, similar to the observations by Delgado et al. (2019). In control animals, *Bacteroidetes* (48.16%) and *Firmicutes* (41.48%) were the predominant phyla, together accounting for the majority of the rumen microbiota (Table 2). Minor phyla included

Table 2: Relative abundance of bacterial phyla in the rumen metagenome of lactating cows in three experiments (% of assigned phyla)

Name of phyla	Control	SARA	PRO
Bacteroidetes	48.16	48.42	54.78
Firmicutes	41.48	22.37	23.01
Proteobacteria	4.372	13.91	9.757
Fibrobacteres	0.4392	6.076	4.288
Spirochaetes	0.8816	2.526	2
Cyanobacteria	1.199	2.098	1.066
Tenericutes	1.01	1.255	1.226
Actinobacteria	0.8816	0.6145	0.6564

Proteobacteria (4.37%), followed by *Cyanobacteria* (1.20%), *Tenericutes* (1.01%), *Spirochaetes* (0.88%), and *Actinobacteria* (0.88%). *Fibrobacteres*, a key fibrolytic phylum, was present at a relatively low abundance (0.44%). These results were consistent with the reports of Sadan et al. (2020), Sato et al. (2021) and Malik et al. (2023) about dairy and crossbred cattle. Although some metagenomic studies had reported higher *Firmicutes* than *Bacteroidetes* (Stewart et al., 2018; Delgado et al., 2019), such variation likely reflected the differences in diet, breed, and geography.

Under SARA conditions, a shift in microbial composition was observed. *Bacteroidetes* (48.42%) remained the dominant phylum; however, *Firmicutes* decreased substantially to 22.37%. *Proteobacteria* increased significantly to 13.91%, indicating dysbiosis. *Fibrobacteres* showed a pronounced increase (6.08%) compared to the control. Other minor phyla, including *Spirochaetes* (2.53%), *Cyanobacteria* (2.10%), and *Tenericutes* (1.26%), also exhibited moderate increases, while *Actinobacteria* decreased to 0.61%. In contrast, Khafipour et al. (2009) reported reduced Gram-negative *Bacteroidetes* during severe SARA, suggesting the sensitivity to pH. The limited phylum-level shifts observed in the present study might reflect the relatively mild SARA condition (pH 5.66–5.8).

After probiotic supplementation, *Bacteroidetes* increased further to 54.78%, suggesting a beneficial modulation of the microbial community. *Firmicutes* slightly increased (23.01%), compared to SARA. *Proteobacteria* decreased to 9.76% compared to SARA, indicating partial recovery from dysbiosis. *Fibrobacteres* (4.29%) remained elevated relative to the control but lower than in SARA. Minor phyla such as *Spirochaetes* (2.00%) and *Tenericutes* (1.23%) were relatively stable, while *Cyanobacteria* (1.07%) decreased toward control levels. *Actinobacteria* remained low (0.66%). Similar findings on probiotic-mediated stabilization of the rumen were obtained by Zhao et al. (2022) and Mei et al. (2023).

3.2.1.2. Family level

Prevotellaceae (29.08%) predominated in the control rumen, followed by *Oscillospiraceae* (12.38%) and *Lachnospiraceae* (12.06%), with moderate occurrence of *Tannerellaceae* (3.40%) and *Sphingobacteriaceae* (3.83%), indicating a balanced fibrolytic-saccharolytic ecosystem. *Prevotellaceae* are involved in starch and protein degradation, whereas *Oscillospiraceae* and *Lachnospiraceae* contribute to cellulose breakdown and butyrate production (Stewart et al., 2018; Sadan et al., 2020).

Under SARA, *Succinivibrionaceae* (11.31%) and *Fibrobacteraceae* (6.92%) increased while *Oscillospiraceae* and *Lachnospiraceae* declined, reflecting an acid-induced microbial shift and enhanced succinate/propionate production, consistent with the previous reports (Khafipour et al., 2009; McCann et al., 2016; Fliegerova et al., 2021).

After probiotic supplementation, *Prevotellaceae* (38.84%) remained dominant, with partial recovery of *Lachnospiraceae* (6.49%) and *Fibrobacteraceae* (4.93%) and reduced *Succinivibrionaceae* (6.56%), indicating the microbial restoration similar to earlier research findings (Pinloche et al., 2013; Rabee et al., 2022; Mei et al., 2023).

3.2.1.3. Genus level

In the control group, *Prevotella* (25.45%) predominated, followed by *Succiniclasticum* (3.99%), *Parabacteroides* (3.81%), and *Anseongella* (3.79%), indicating a stable and functionally diverse rumen microbiota (Table 3). *Prevotella* plays a central role in starch, pectin, and protein degradation, while *Succiniclasticum* converts succinate to propionate. The dominance of *Prevotella* in healthy rumen has been widely reported (Stewart et al., 2018; Sadan et al., 2020; Malik et al., 2023).

Table 3: Relative abundance of bacterial genera in the rumen metagenome of lactating cows on three experiments (% of assigned genera)

Name of genera	Control	SARA	PRO
Prevotella	25.45	26.85	34.26
Succinivibrio	1.522	7.682	5.79
Fibrobacter	0.5906	7.539	5.408
Parabacteroides	3.81	3.802	4.94
Succiniclasticum	3.985	1.998	2.171
Porphyromonas	2.955	2.834	1.894
Anseongella	3.795	1.752	1.505
Butyrivibrio	3.089	1.692	1.547
Paraprevotella	2.151	1.65	2.316
Treponema	1.015	2.855	2.241
Saccharofermentans	3.493	1.151	1.06
Ruminobacter	0.0958	4.409	1.07
Ruminococcus	1.04	1.229	1.01

Under SARA, *Succinivibrio* (7.68%), *Fibrobacter* (7.54%), and *Ruminobacter* (4.41%) increased, whereas *Prevotella* (26.85%) remained predominant (Figure 2). The enrichment of *Succinivibrio* and *Ruminobacter* reflected the adaptation to low-pH and high-fermentable carbohydrate conditions (Khafipour et al., 2009; Plaizier et al., 2017). The rise in *Fibrobacter* might indicate a compensatory response to maintain fibre degradation under acid stress, contrasting with Fernando et al. (2010), who reported the presence of reduced fibre digesters under high-energy diets.

In the probiotic-fed group, *Prevotella* (34.26%) remained dominant, with moderate levels of *Succinivibrio* (5.79%), *Ruminobacter* (1.07%) and *Fibrobacter* (5.41%). This

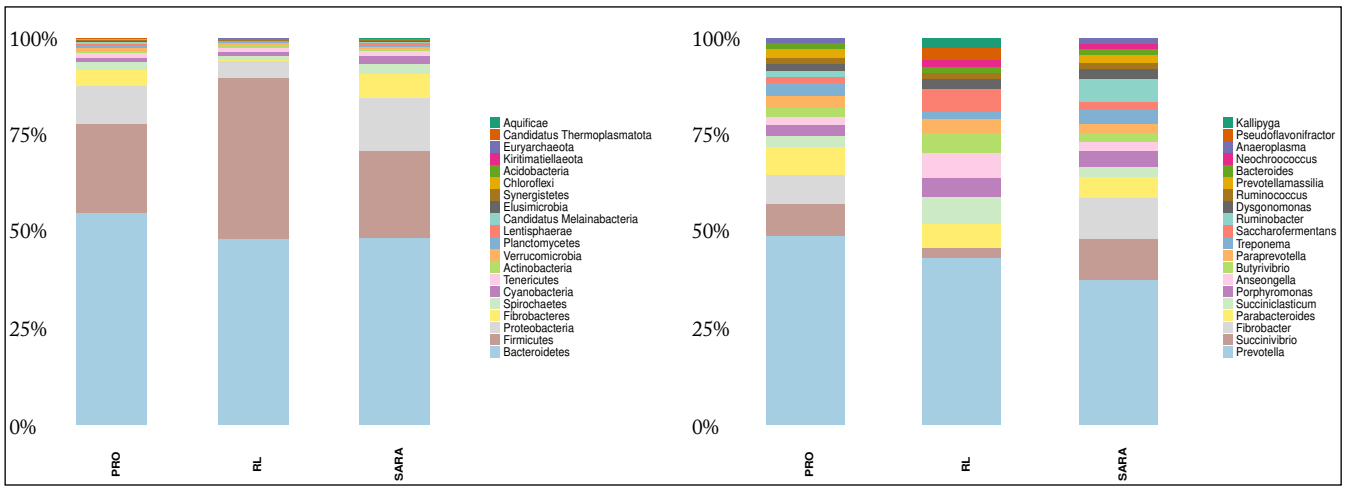

Figure 2: Bar plot for relative abundance of bacterial phyla and genera across three experimental groups, (% of assigned genera)

balanced distribution suggested the restoration of rumen stability, consistent with the reports of probiotic-mediated fibrolytic recovery and reduced dysbiosis (Pinloche et al., 2013; Rabee et al., 2022; Mei et al., 2023).

3.2.1.4. Species level

In the control group, *Prevotella ruminicola* (4.69%), *Succiniclasticum ruminis* (4.81%), *Anseongella ginsenosidimutans* (4.58%), and *Saccharofermentans acetigenes* (4.22%) predominated, indicating a balanced rumen microbiota; *P. ruminicola* degrades starch and peptides, while *S. ruminis* converts succinate to propionate (Stewart et al., 2018; Sadan et al., 2020).

Under SARA, *Succinivibrio dextrinosolvens* (9.22%), *Fibrobacter succinogenes* (9.02%), *Ruminobacter amylophilus* (5.29%), and *P. ruminicola* (5.69%) increased, reflecting an adaptation to high-carbohydrate-associated acidic conditions and a compensatory fibrolytic response (Khafipour et al., 2009; Plaizier et al., 2017).

After probiotic supplementation, *P. ruminicola* (7.50%) remained dominant with moderate levels of *S. dextrinosolvens* (6.88%) and *F. succinogenes* (6.42%), indicating microbial stabilization and improved fibre degradation, consistent with the probiotic-mediated rumen homeostasis (Pinloche et al., 2013; Mei et al., 2023).

3.2.2. Bacterial diversity analysis

Alpha diversity indices showed the highest diversity in control (RL) (Shannon=5.011; Simpson=0.984), intermediate in the probiotic group (4.711; 0.976), and lowest in SARA (4.632; 0.970), indicating reduced richness and evenness under acidotic conditions. The higher diversity in controls agreed with Hagey et al. (2022). The decline in SARA concurred with Plaizier et al. (2017), Khafipour et al. (2009), and Liu et al. (2025), who reported reduced diversity during grain-induced acidosis due to proliferation of acid-tolerant taxa and loss of fibrolytic bacteria. The relatively greater diversity in the probiotic group aligned with Fomenky et al. (2018) and Zhao et al. (2022), indicating the stabilization of rumen pH and maintenance of microbial diversity. Overall, SARA impaired microbial diversity, whereas probiotic supplementation promoted a more resilient rumen ecosystem.

Beta diversity based on Bray-Curtis ' dissimilarity (Table 4) showed clear separation among the control (RL), SARA, and probiotic (PRO) groups in the heatmap and Principal coordinate analysis (PCoA). The greatest dissimilarity was observed between the control and PRO (0.3955), followed by the control and SARA (0.3825), while SARA and PRO showed comparatively lower dissimilarity (0.3577), likely reflecting the pre- and post-supplementation samples from the same animals. These findings agreed with Khafipour et al. (2009) and Plaizier et al. (2017), who reported pronounced microbial restructuring during SARA, and with Fomenky et al. (2018), who observed partial moderation of diet-induced shifts following probiotic supplementation. Although Liu et al. (2025) noted a possible overlap depending on host and sampling factors, the present results demonstrated distinct microbial community structures across all the treatments.

The heatmap of Bray-Curti's dissimilarity (Figure 3) showed low within-group variation (blue diagonal) and clear between-group differences. The strongest dissimilarity was observed between the control and probiotic groups, followed by control and SARA, while SARA and probiotic groups showed moderate dissimilarity. The dendrogram supported this pattern, with SARA and probiotic samples clustered together and the control group formed a distinct branch, indicating a unique microbial composition.

Principal coordinate analysis (PCoA) (Figure 4) based on Bray-Curtis dissimilarity revealed clear separation among

Table 4: Beta diversity measures of microbial communities among the experimental groups: control (RL), SARA and PRO

Samples	PRO	Control (RL)	SARA
PRO	0	0.39545659	0.35765556
Control (RL)	0.39545659	0	0.38252981
SARA	0.35765556	0.38252981	0

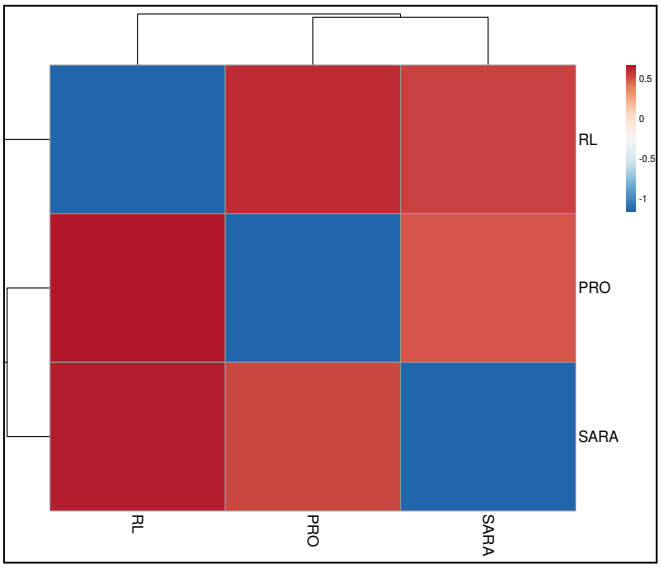

Figure 3: Bray-curtis dissimilarity heatmap representing beta diversity of microbial communities among control (RL), SARA and PRO

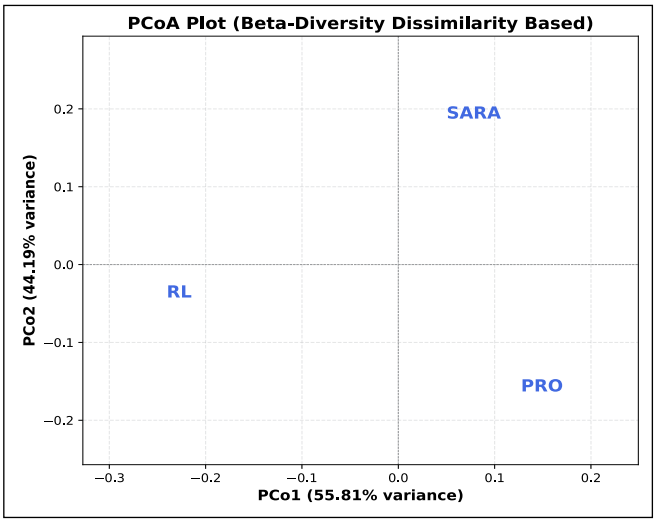

Figure 4: Principal coordinate analysis (PCoA) plot of microbial communities in control (RL), SARA and PRO

the control, SARA, and probiotic groups, with PCo1 (55.81%) and PCo2 (44.19%) explaining >99% of the total variation. SARA samples formed a distinct cluster, indicating dysbiosis under low ruminal pH, whereas the probiotic group shifted away from the SARA profile but remained distinct from the control. Control samples clustered independently, representing the most divergent microbial structure. The results were consistent with Plaizier et al. (2017), who reported microbial restructuring during SARA. The intermediate clustering of the probiotic group suggested partial microbial restoration, in alignment with the findings of Fomenky et al. (2018) and Rabee et al. (2022). The present results were also in agreement with Pinloche et al. (2013), who demonstrated that *S. cerevisiae* improved rumen pH and modulated microbial composition under SARA conditions.

Krona plots (Figures 5A, 5B, 5C) showed clear taxonomic shifts among the groups. Control cows had a balanced microbiota dominated by *Bacteroidetes* and *Firmicutes*, with *Prevotella* and fibrolytic taxa such as *F. succinogenes*, consistent with stable rumen fermentation (Ozbayram et al., 2018). SARA reduced *Firmicutes* and increased *Proteobacteria*, particularly acid-tolerant *S. dextrinosolvens*, indicating ruminal dysbiosis under low pH (Khafipour et al., 2009; McCann et al., 2016; Fliegerova et al., 2021). Probiotic supplementation partially restored the balance, increasing *Firmicutes*, *Prevotella*, *Ruminococcaceae*, and *Lachnospiraceae*, supporting the recovery of a functional microbiome (Rabee et al., 2022; Mao et al., 2023).

The Venn diagram (Figure 5D) revealed 1763, 1860, and 1548 microbial species in the control, SARA, and probiotic groups, respectively. A total of 1029 species were shared across all the groups, indicating a substantial core rumen microbiome. The presence of unique species, numbered 394 in control, 400 in SARA, and 216 in the probiotic group, demonstrated that each treatment also maintained distinct microbial populations alongside the shared community.

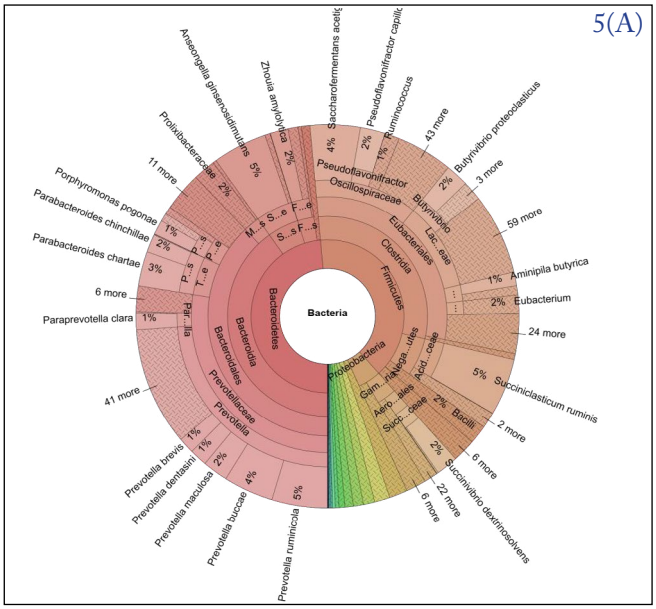

Figure 5: Continue...

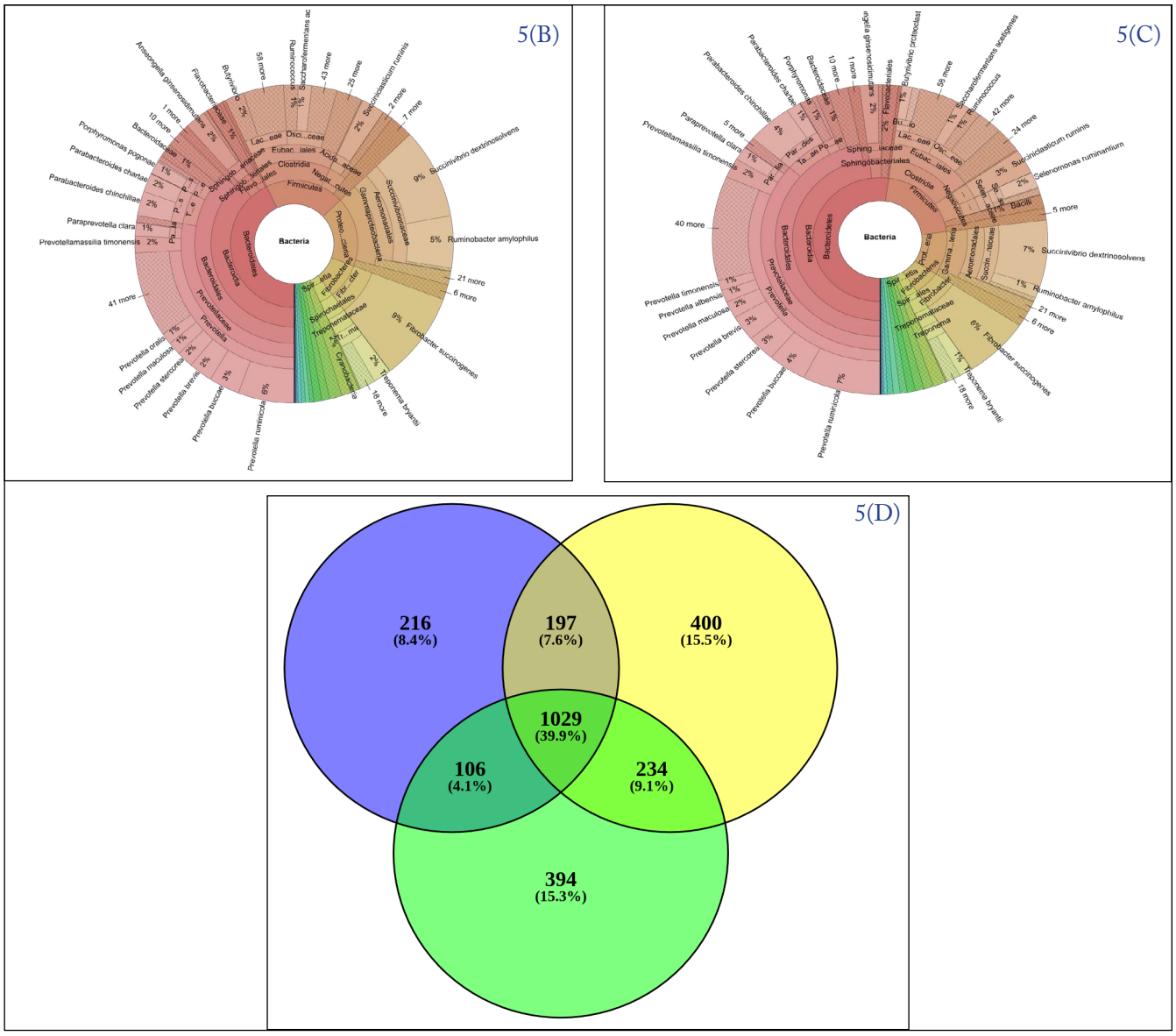

Figure 5(A): Krona plot showing the taxonomic distribution of microbial communities in control (RL) group; Figure 5(B): Krona plot showing the taxonomic distribution of microbial communities in SARA group; Figure 5(C): Krona plot showing the taxonomic distribution of microbial communities in PRO group; Figure 5(D): Venn diagram depicting the shared and unique species among experimental groups control (RL), SARA and PRO

4. CONCLUSION

SARA reduced rumen pH and induced microbial dysbiosis, characterised by decreased fibrolytic *Firmicutes*, increased acid-tolerant *Proteobacteria*, reduced diversity, and impaired rumen stability. It altered rumen physiology and microbiology, with shifts in dominant taxa, reduced richness and evenness, and distinct core microbiomes. Probiotic supplementation improved pH, enhanced beneficial fibrolytic taxa, reduced dysbiosis-associated bacteria, and partially restored microbial homeostasis and core communities. Hence, it is the need of the hour to conduct more research to identify microorganisms having greater probiotic potential.

5. ACKNOWLEDGEMENT

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