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Molecular profiling of major carbohydrate utilising rumen bacteria in lactating cows with sub-acute rumen acidosis

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Abstract

Sub-acute rumen acidosis (SARA) is a major nutritional disorder in high producing cattle, arising from excessive concentrate feeding and characterised by prolonged rumen pH depression and microbial dysbiosis. The rumen microbiome plays a central role in fibre degradation, carbohydrate fermentation, and metabolic homeostasis; therefore, PCR detection of the major carbohydrate utilising rumen bacteria and understanding the potential corrective effects of probiotics is essential for improving rumen health. The present study investigated key rumen bacterial populations in clinically healthy (control), SARA affected, and probiotic supplemented cows using PCR based detection. The Rumen pH values in stall fed cattle (5.2 to 5.8) confirmed SARA, while probiotic supplementation elevated pH toward normal physiological levels. PCR screening detected Butyrivibrio, Ruminococcus, Prevotella, and Fibrobacter sp. across all groups, reaffirming their status as core rumen bacteria. Sequencing of representative amplicons validated target specificity. Overall, the study demonstrated that SARA in cattle is marked by reduced rumen pH while probiotic supplementation helped restore pH toward normal range and supported the presence of core fibre degrading genera. PCR detection and sequence confirmation of Butyrivibrio, Ruminococcus, Prevotella, and Fibrobacter sp. across all groups highlighted their fundamental role in rumen function. These findings reinforced the importance of maintaining microbial balance to prevent SARA and promote optimal rumen health.

Keywords: Sub-acute rumen acidosis, Butyrivibrio, Fibrobacter, Ruminococcus, Prevotella

The rumen microbiome is a highly dynamic community that drives the digestion and fermentation of fibrous and non-fibrous carbohydrates, proteins and lipids, supplying energy and essential nutrients to the host while supporting immune function and maintaining rumen epithelial integrity (Liu et al., 2021). In a healthy state, diverse microbes work synergistically to produce volatile fatty acids and regulate rumen pH. However, dietary imbalances, particularly excessive concentrate feeding can disturb this equilibrium and lead to subacute ruminal acidosis (SARA), a condition marked by prolonged reduction in rumen pH (5.2-5.8) (Li et al., 2013), suppression of fibre degrading bacteria (Fernando et al., 2010), and proliferation of acid tolerant species (Khafipour et al., 2009). SARA affects a significant proportion of lactating

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cattle and is associated with reduced feed intake, milk fat depression, digestive upset, inflammation, and loss of productivity.

Probiotics have gained focus as a practical intervention for restoring microbial balance, stabilising rumen pH, and enhancing fibre utilisation (Mei et al., 2023). The present study was therefore undertaken to identify the major carbohydrate utilising bacteria *viz*. *Butyrivibrio* sp., *Fibrobacter* sp., *Ruminococcus* sp. and *Prevotella* sp. in rumen liquor associated with SARA by polymerase chain reaction (PCR).

Materials and methods

Sample collection

Lactating crossbred cows at the University Livestock Farm and Fodder Research and Development Scheme (ULF & FRDS), Mannuthy, were used for this study. All animals were maintained under uniform feeding and management. Rumen liquor was collected from six healthy cows and six SARA affected cows (pH ≤ 5.8) before and after probiotic supplementation using a flexible stomach tube connected to a vacuum pump, positioned in the ventral sac of the rumen. About 500 mL of whole rumen contents were collected, after discarding the first 200 mL to reduce saliva contamination (Sadan et al., 2020). Samples were transported immediately to the Department of Veterinary Microbiology, filtered through sterile muslin cloth, and stored at –80°C until analysis.

pH of the rumen liquor

The pH of rumen liquor was measured immediately after collection using the pH meter in the Department of Veterinary Microbiology, CVAS, Mannuthy.

Probiotic supplementation

The SARA affected cows were supplemented daily with a commercial probiotic bolus @ two bolus twice daily for a period of two weeks. Each bolus contained *Saccharomyces cerevisiae* (10^9 CFU/bolus), *Lactobacillus sporogenes* (10^6 CFU/bolus) and *Aspergillus oryzae* (10^6 CFU/bolus).

Statistical analysis

All statistical analyses were conducted using statistical product and service solutions (SPSS) software, version 24.0.

DNA extraction and purification

The DNA were extracted from the rumen liquor samples using Qiagen QIAamp fast DNA stool mini kit following manufacturer's instructions with slight modification. The quality of the extracted DNA was tested by UV spectrophotometric analysis using Nanodrop TM 2000C. The DNA samples that exhibited satisfactory purity were subjected to PCR to detect major rumen bacterial groups using species specific gene targets. *Butyrivibrio* sp., *Ruminococcus* sp. and *Prevotella* sp. were identified using primers targeting the 16S rRNA gene while *Fibrobacter* sp. were detected through amplification of the cellulase gene.

Design of primers and standardisation of polymerase chain reaction

The primers were designed to amplify the 16S rRNA genes of *Butyrivibrio* sp., *Ruminococcus* sp. and the cellulase gene of *Fibrobacter* sp., using Primer3 and the Primer-BLAST tool available on the NCBI website and published primers were used for *Prevotella* species targeting 16S rRNA. All designed primers were targeted for amplicon sizes below 500 bp. The stability of each primer was evaluated using the Sequence Manipulation Suite (SMS), and the primers were subsequently custom synthesised.

The protocols for standardisation of PCR are listed in Table 2. Each PCR reaction was carried out in a total volume of 20 µL containing 10 µL of 2X EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Japan), 1 µL each of forward and reverse primers (10 pmol/µL), 2 µL of template DNA and nuclease free water (NFW) to make up the final volume.

Initial optimisation of PCR conditions was carried out using gradient PCR in Mini thermal cycler (Bio Rad, USA) using extracted DNA as template. A series of

Table 1. Primers for the amplification of major carbohydrate-utilising bacteria in the rumen

Primers	Gene	Sequence (5'- 3')	Amplicon size (bp)
BUT-F	16S rRNA	CTGACGTTGAGGCTCGAAGG	494
BUT-R		CGACATTGTAGCACGTGTGT	
FIB-F	Cellulase	AACGACCGATGTTACCGACT	321
FIB-R		TGACCAACTGCAGCACTAGA	
RUM-F	16S rRNA	TCTGGAAACGGATGGTA	295
RUM-R		CCTTTAAGACAGGAGTTTACAA	
PRE-F	16S rRNA	GGTTATCTTGAGTGAGTT	486
PRE-R		CTGATGGCAACTAAAGAA	

Table 2. Protocols for standardisation of 16S rRNA gene of *Butyrivibrio* sp., *Ruminococcus* sp., *Prevotella* sp. and Cellulase gene of *Fibrobacter* sp.

Steps			Temperature (°C)	Time
Initial denaturation			95	3 min.
34 cycles	Denaturation		95	30 sec.
	Annealing	*Butyrivibrio* sp.	60 to 70	30 sec.
		Fibrobacter sp.	57.8 to 67.8	
		Ruminococcus sp.	52 to 62	
		Prevotella sp.	45.8 to 55.8	
	Extension		72	1 min.
Final extension			72	5 min.

Table 3. 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

temperature gradients were tested to determine the optimal annealing temperatures for amplifying the target genes of the selected rumen bacteria. The annealing temperatures that yielded the best amplification results were selected for all the respective template DNA samples for PCR. Thus, all the extracted DNA samples were tested specifically for the four bacterial species mentioned.

The PCR products, controls (5 µL), and DNA ladder (3 µL) were electrophoresed on agarose gel at 80 V, visualised under UV, documented (Bio-Rad, USA), and the amplicons were sequenced and analysed.

Nucleotide sequence analysis

The amplicons from each of the PCRs were sequenced at GeneSpec Private Limited, India, using Sanger's dideoxy nucleotide chain termination method. Both forward and reverse primers were used for bidirectional sequencing. The obtained sequence of each gene product was aligned and NCBI BLAST tool was used to analyse the similarity of the products obtained with other published sequences available on the NCBI database.

Results and discussion

pH of the rumen liquor

The pH of the rumen liquor samples measured using the pH meter in the Department of Veterinary Microbiology is given in Table 3.

The measured pH of the rumen liquor for SARA-affected animals were in the range of 5.6 to 5.8. Garret et al. (1999) reported that the pH value of 5.5 is considered as the most reliable cut-off point to differentiate between SARA (pH < 5.5) and SARA negative animals (pH > 5.8). Dohme et al. (2008) and Li et al. (2013) had also noticed similar findings. In the present study, animals having rumen liquor pH ≤ 5.8 was considered as SARA affected.

The rumen pH of SARA affected cattle was significantly different and lower than that of the healthy control group (t = 6.194, p = 0.002) (Table 4). This indicated a statistically strong difference, confirming the presence of SARA, as rumen pH < 5.8 for several hours is characteristic of the condition.

Probiotic supplementation resulted in a significant increase in rumen pH in SARA affected cattle (t = 6.508, p = 0.001) (Table 5). The pH increased from an acidic level consistent with SARA (5.75) to a near-physiological range (~6.9), indicating a substantial improvement in rumen environment and partial recovery from acidosis.

Bach et al. (2007) and Chung et al. (2011) reported that *Saccharomyces cerevisiae* supplementation improved rumen pH and reduced lactic acid in cattle with SARA. In the present study, probiotics similarly produced

Table 4. Statistical analysis of the pH of rumen liquor samples collected from healthy and SARA affected cows

	Before probiotic	**t- value**	**p- value**
SARA	5.7550 ± 0.02487	6.194**	0.002
Control	7.8233 ± 0.33297		

** Means are significantly different at 0.01 level

Table 5. Statistical analysis of the pH of the rumen liquor samples collected from SARA affected cows and probiotics supplemented cows

	Before probiotic	After probiotic	t- value	p- value
SARA	5.7550 ± 0.02487	6.9167 ± 0.16212	6.508**	0.001

a significant rise in rumen pH (p = 0.001, t = 6.508), increasing from 5.75 ± 0.02 before supplementation to 6.91 ± 0.16 afterwards. This improvement reflected restored buffering capacity and more stable fermentation, likely due to enhanced lactate utilisation and the recovery of beneficial fibrolytic bacteria. Overall, the findings confirmed that probiotics effectively mitigate rumen dysbiosis and supported fermentation efficiency in SARA affected cattle.

***Detection of Butyrivibrio* sp.*, Fibrobacter* sp.*, Ruminococcus* sp. *and Prevotella* sp.**

The PCR protocols were standardised using DNA from a cow in the control group. Gradient PCR identified optimal annealing temperatures for *Butyrivibrio*, *Fibrobacter* and *Ruminococcus* species. Clear amplicons were obtained at 63.8°C for *Butyrivibrio* (494 bp), 59.8°C for *Fibrobacter* (321 bp) and 55.8°C for *Ruminococcus* (295 bp). For *Prevotella* gradient PCR within 45.8-55.8°C established 53.8°C as the optimal annealing temperature for *Prevotella*.

A total of 18 rumen liquor samples (six healthy, six SARA-affected, and six probiotic-supplemented animals) were screened using PCR targeting the 16S rRNA gene, and *Butyrivibrio* sp. were detected in all samples, confirming their ubiquitous presence across physiological states. This agrees with earlier findings of Tajima et al. (1999) where they reported consistent *Butyrivibrio* sequences in bovine rumen clone libraries. Zhu et al. (2014) also observed diverse *Butyrivibrio* populations in goats, highlighting the genus as a core fibrolytic group in ruminants. However, studies on dietary stress showed that its abundance can shift. Fernando et al. (2010) noted that high-grain diets reduced fibrolytic bacteria, including *Butyrivibrio*, indicating that although the genus remained detectable, its population can decline under SARA or acidic rumen conditions.

In the present study, PCR detection of the 16S rRNA gene of *Ruminococcus* sp. showed their presence in five out of six control samples and in all SARA affected and probiotic supplemented animals, indicating that the genus remained stable or even increased under stress or supplementation. Previous research similarly reported *Ruminococcus* as a consistently maintained cellulolytic group in the rumen, with *Ruminococcus albus* and *Ruminococcus flavefaciens* identified as dominant species (Krause et al., 1999) and higher abundance linked to solid feed intake (Wang et al., 2017). However, Bi et al. (2018) found reductions in *Ruminococcus* under high-grain diets, suggesting decreased competitiveness in low fibre conditions. Overall, the consistent detection across all groups supports their role as key fibre degraders, while showing that detection does not always reflect stable abundance under high-concentrate feeding.

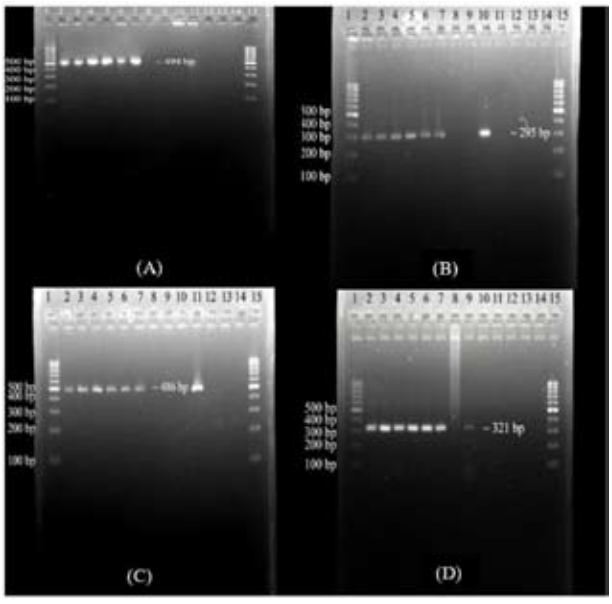

Fig. 1. Agarose gel electrophoresis depicting amplicons - SARA group

(A) 16S rRNA genes of *Butyrivibrio* sp. (~494 bp): L1- 100 bp ladder, L2 to 7- Samples, L10- Positive control, L11- Negative control **(B)** 16S rRNA genes of *Ruminococcus* sp. (~295 bp): L1- 100 bp ladder, L2 to 7- Samples, L10- Positive control, L11- Negative control **(C)** 16S rRNA genes of *Prevotella* sp. (~486 bp): L1- 100 bp ladder, L2 to 7- Samples, L11- Positive control, L12- Negative control **(D)** Cellulase gene of *Fibrobacter* sp. (~321 bp): L1- 100 bp ladder, L2 to 7- Samples, L9- Positive control, L10- Negative control.

The PCR targeting the 16S rRNA gene of *Prevotella* sp. produced positive amplicons in five control samples and in all samples from the SARA affected and probiotic supplemented groups, indicating strong adaptability of this genus across varied rumen conditions. This finding agreed with Stevenson and Weimer (2007), who reported *Prevotella* as a dominant and consistently detected genus in bovine rumen clone libraries, and with Bekele et al. (2010), who observed its high prevalence in cattle fed diets with different forage to concentrate ratios. In contrast, Petri et al. (2013) noted that although *Prevotella* remained detectable during SARA, its population could decline under severe dietary stress, showing that presence does not always equate to ecological dominance.

The PCR targeting the cellulase gene of *Fibrobacter* sp. detected amplicons in all samples from the control, SARA affected, and probiotic supplemented groups, indicating that this genus persisted stably in the rumen regardless of dietary stress. This agreed with earlier reports showing that *Fibrobacter succinogenes* is one of the most consistently detected cellulolytic species

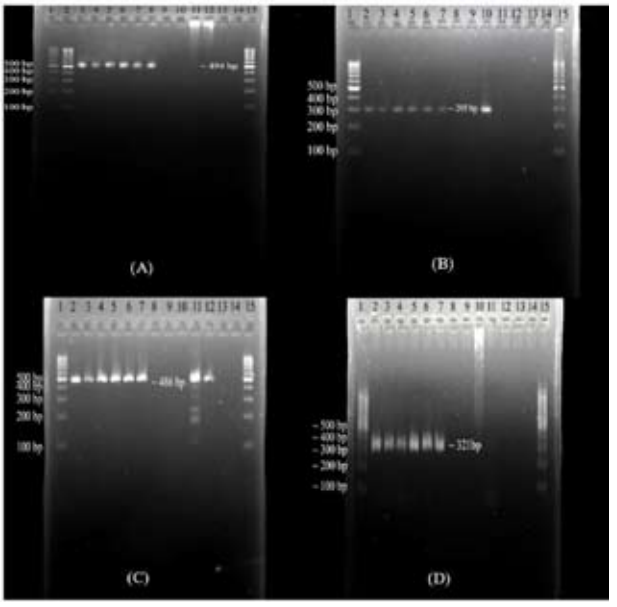

Fig. 2. Agarose gel electrophoresis depicting amplicons-after probiotic supplementation

(A)16S rRNA genes of *Butyrivibrio* sp. (~494 bp): L2- 100 bp ladder, L3 to 8- Samples, L11- Positive control, L10- Negative control **(B)** 16S rRNA genes of *Ruminococcus* sp. (~295 bp): L1- 100 bp ladder, L2 to 7- Samples, L10- Positive control, L11- Negative control **(C)** 16S rRNA genes of *Prevotella* sp. (~486 bp): L1- 100 bp ladder, L2 to 7- Samples, L11- Positive control, L12- Negative control **(D)** Cellulase gene of *Fibrobacter* sp. (~321 bp): L1- 100 bp ladder, L2 to 7- Samples, L10- Positive control, L11- Negative control

in ruminants. Krause et al. (1999) and Tajima et al. (2001) identified *Fibrobacter* as a dominant fibrolytic taxon across cattle fed different diets. In contrast, Fernando et al. (2010) found that severe SARA induced by abrupt high grain feeding reduced the counts of *Fibrobacter*, suggesting that although PCR detection remained reliable, population density may decline under extreme ruminal acidosis. Overall, the present findings indicated that *Fibrobacter* sp. were resilient and functionally important, even though their population could fluctuate with diet severity.

The distribution of major carbohydrate utilising rumen bacteria detected across different sample types is summarised in Table 6.

Nucleotide sequence analysis

Representative PCR amplicons of *Butyrivibrio* sp., *Ruminococcus* sp., *Prevotella* sp. and *Fibrobacter* sp. and which yielded specific bands of 494 bp, 295 bp, 486 bp and 321 bp, respectively, were sequenced using Sanger's dideoxy chain termination method at GeneSpec Pvt. Ltd., India. The forward and reverse reads were assembled using EMBOSS Merger and the resulting consensus sequences were subjected to BLASTn analysis which confirmed their identity with previously reported sequences. The validated nucleotide sequences were subsequently submitted to the NCBI GenBank database and accession numbers were obtained (Table 7).

Conclusion

This study demonstrated that SARA in lactating crossbred cows was associated with a significant depression of rumen pH ($p = 0.002$), confirming the establishment of an acidotic rumen environment, while probiotic supplementation effectively restored rumen pH

Table 6. Detection of *Butyrivibrio* sp., *Fibrobacter* sp., *Ruminococcus* sp. and *Prevotella* sp. by PCR in rumen liquor samples from control, SARA and Probiotic fed animals

Treatment	Samples tested	*Butyrivibrio* sp. positive	*Fibrobacter* sp. positive	*Ruminococcus* sp. positive	*Prevotella* sp. positive
Healthy	6	6	6	5	5
SARA	6	6	6	6	6
Probiotic	6	6	6	6	6

Table 7: Details of accession numbers obtained for 16S rRNA gene of *Butyrivibrio* sp., *Ruminococcus* sp. and *Prevotella* sp.

Sl. No.	Sample ID	Groups and Bacteria	Genbank Accession No.
1	CB-BF-BR	Control- *Butyrivibrio* sp.	PX560446
2	CR-RF-RR	Control- *Ruminococcus* sp.	PX559135
3	CP-PF-PR	Control- *Prevotella* sp.	PX578197
4	SB-BF-BR	SARA- *Butyrivibrio* sp.	PX559138
5	SR-RF-RR	SARA- *Ruminococcus* sp.	PX559139
6	SP-PF-PR	SARA- *Prevotella* sp.	PX578199
7	PB-BF-BR	Probiotic- *Butyrivibrio* sp.	PX559136
8	PR-RF-RR	Probiotic- *Ruminococcus* sp.	PX559137
9	PP-PF-PR	Probiotic- *Prevotella* sp.	PX578198

towards physiological levels. The marked increase in pH after probiotic feeding indicated improved rumen buffering capacity and stabilization of fermentation patterns. PCR-based detection revealed that major carbohydrate-utilising bacteria, namely *Butyrivibrio* sp., *Fibrobacter* sp., *Ruminococcus* sp. and *Prevotella* sp., were consistently present in healthy, SARA-affected and probiotic-supplemented animals, highlighting their fundamental role in rumen metabolism. Although their presence was maintained even under acidotic conditions, earlier reports and the present findings together suggest that SARA may primarily influence their abundance and functional efficiency rather than complete elimination. Since polymerase chain reaction was employed primarily to detect the presence of major carbohydrate-utilising bacteria, the present study could not quantify their relative abundance. Therefore, advanced metagenomic approaches would be valuable in future investigations to precisely evaluate changes in the abundance and dynamics of specific bacterial populations during SARA and following probiotic supplementation. Sequence analysis and BLAST confirmation validated the specificity of the PCR assays and established the genetic identity of the detected rumen bacteria, with successful submission of representative sequences to GenBank further strengthening the molecular evidence. Overall, the results confirmed that probiotic supplementation containing *Saccharomyces cerevisiae*, *Lactobacillus sporogenes* and *Aspergillus oryzae* was effective in alleviating ruminal acidosis by improving rumen pH and supporting a favourable microbial environment. This study highlighted the utility of PCR as a rapid and reliable tool for monitoring key rumen bacterial populations and supports the strategic use of probiotics as a practical approach for mitigating SARA and improving rumen health and productivity in dairy cattle.

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Conflict of interest

The authors declare that they have no conflict of interest.

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