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Research Article

The Effects of Dry Yeast Supplementation on Rumen Ciliated Protozoa Population and Volatile Fatty Acid Production in Lactating Beef Cows

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ABSTRACT

This present study aims to assess the impact of supplementing dry yeast (*Saccharomyces cerevisiae*: SC) on ruminal ciliated protozoa and volatile fatty acid production in lactating beef cows. Nine lactating beef cows were divided into three groups: the control group (SC0) received a 16% protein commercial concentrate diet without yeast supplementation at 0.5% of body weight (BW) in kilograms, while the second (SC2) and third (SC4) groups were given the basal diet along with 2 or 4 grams per head/day of SC, respectively, plus 10% BW roughage for 21 days. At the last day of experiment, ruminal fluid was collected at 2 time points (0 and 4 h post-feeding) using rumenocentesis technique. The samples underwent analysis for ruminal pH, ciliated protozoa motility and population, and volatile fatty acids production. Results demonstrated that the SC2 group exhibited a lower pre-feeding ruminal pH compared with the other groups ($P < 0.05$), whereas the SC4 group displayed reduced protozoa motility ($P < 0.05$). Although the SC2 group tended to have a higher ciliated protozoa population, this difference was not statistically significant. Total VFA production was significantly elevated in the SC4 group ($P < 0.05$), and both the SC2 and SC4 groups exhibited increased total VFAs and acetate levels post-feeding. The SC2 group displayed significantly higher acetate:propionate and acetate:butyrate ratios compared to the other groups ($P < 0.05$). These findings suggest that dry yeast supplementation can modulate rumen fermentation through alterations in protozoal activity and VFA profiles, with potential implications for digestive efficiency and nutrient absorption in lactating beef cows.

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1. Introduction

During the lactating period, beef cows require high energy to support the milk production process for 6-7 months of calf raising. This phase is considered the most stressful for beef cows as the milk contains both high levels of fat and protein to support calf growth. Changes in body condition score as a marker of energy imbalance have been reported to negatively affect reproductive performances in postpartum cows (Humblot *et al*., 2008). Providing high-quality nutrition to beef cows during this critical period is essential for optimizing feed utilization and enhancing nutrient absorption, which helps maintain optimal body condition scores, supports substantial milk production, and promotes reproductive function. Feed supplementation, particularly with yeast, has been demonstrated to improve digestive efficiency and nutrient absorption, thereby contributing to increased milk yield, the prevention of metabolic disorders, and enhanced reproductive performance in dairy cows (Szucs *et al*., 2013; Kalmus *et al*., 2019). However, the traditional practice of free-range grazing for beef cattle in Thailand often leads farmers to neglect proper nutrition management, including the potential benefits of yeast supplementation, which are crucial to meeting the cows' nutritional needs and sustaining their gut health during lactation.

The cattle rumen serves a pivotal function as the organ responsible for synthesizing volatile fatty acids (VFAs), including acetate, propionate, and butyrate, through microbial fermentation (France and Siddons,1993). These VFAs act as the primary energy source for ruminants, contributing to around 70% of their total energy requirements. The quantity of each VFA produced is influenced by dietary composition (Moran, 2005). In the realm of rumen ecology, a diverse range of microorganisms, including cellulolytic bacteria, lactate-producing bacteria, and ciliate protozoa, are commonly found. Each type of microorganism plays a specific role in fermenting and breaking down various nutrients (Castillo-González *et al*., 2014). Notably, rumen ciliated protozoa are recognized for their fibrolytic activity, contributing to 19-28% of the cellulase activity within the total fibrolytic activity in the rumen (Patel and Ambalam, 2008). Their significant role of microorganisms in rumen function is evident in their association with feed digestion and fermentation, as well as in their impact on nitrogen utilization efficiency and methanogenesis (Newbold *et al*., 2015; Park *et al*., 2021). In normal cattle, the pH value typically falls within the range of 6.0 to 7.0. Variation in ruminal pH is a crucial factor that influences the population of microorganisms and the fermentation processes within the rumen, thereby affecting the production of volatile fatty acids (VFAs) and methane. Consequently, maintaining a balanced acid-base environment in the rumen is essential for the overall well-being of cattle (Dijkstra *et al*., 2020).

Saccharomyces cerevisiae (SC) is commonly used as a probiotic supplement in ruminant feed to reduce the need for antimicrobial drugs. These yeasts can proliferate in the digestive system of animals, aiding in the fermentation and digestion of carbohydrates and cellulose within the rumen. The benefits of supplementing live yeast in cattle feed, especially in dairy cows, have been thoroughly studied (Kowalik *et al*., 2012; Marden *et al*., 2008). Previous research suggests that live yeast supplementation within the range of 2-5 grams per individual per day plays a role in regulating rumen acidity (Ambriz-Vilchis *et al*., 2017). This supplementation also positively influences rumen fermentation processes, promoting the production of propionate. This enhancement contributes to the efficient utilization of starch as an energy source. Furthermore, the incorporation of commercial dry yeast at a dosage ranging from 2 to 10 grams per cow per day in the diet of dairy cows has been shown to enhance milk yield and improve milk composition (Kalmus *et al*., 2019; Besseboua *et al*., 2020). Additionally, it reduces somatic cell count, which is indicative of improved udder health, and decreases the concentrations of beta-hydroxybutyrate (BHB) and free fatty acids (FFA) in the blood, which are associated with a reduced risk of ketosis (Kalmus *et al*., 2009; Yalcin *et al*., 2011; Szucs *et al*., 2013). Williams *et al.* (1991) and Ding *et al.* (2014) conducted a study on supplementing yeast in beef cattle. The results of these studies demonstrated an enhancement in the efficiency of roughage digestion in the rumen, along with an increase in the population of beneficial microorganisms in the

rumen. We hypothesize that dietary yeast supplementation in lactating beef cows in tropical regions will enhance digestion and increase volatile fatty acid production, thereby improving nutrient utilization for milk production and ultimately benefiting the calves. These processes may differ from those observed in dairy cows, as current literature on the use of dry yeast in lactating beef cows, particularly its effects on ruminal microbiota and metabolic processes, remains relatively limited. Therefore, this study aims to address this gap by investigating the impact of SC supplementation on ruminal ciliated protozoa populations and VFA production. It will provide insights into the potential of SC as an alternative probiotic, supporting fiber digestion and rumen pH balance, thereby enhancing digestive efficiency and nutrient absorption in lactating beef cows.

2. Materials and Methods

2.1 Animals and Experimental designs

The research focused on Brahman-crossbred beef cows, aged 4 to 7 years and calved within the past 30 days. The nine cows, with an average weight of 349.55 ± 37.20 kilograms, were individually housed in open-sided barn pens at the Srivijaya Agricultural Development Center in Pathio District, Chumphon Province. Prior to the experiment, all cows received vitamin AD3E injections and Ivermectin-F treatment. The animals were randomly assigned to three experimental groups. Cows received fresh Napier grass ad libitum, representing 10% of their body weight (BW) daily, and a single daily meal of pelleted feed containing 16% crude protein, comprising 0.5% of their BW. The chemical composition of the basal diets (concentrate and roughage) fed in the experiment based on dry matter is represented in Table 1. Each group was administered varying levels of commercial dry yeast supplementation (*Saccharomyces cerevisiae*, $20x10^7$ cells/gram, RumenYeast®, ICC, Santa Maria, Brazil) at doses of 0g (SC0), 2g (SC2), or 4g (SC4) per individual per day. The 21-day experiment included a 14-day adaptation period and a 7-day sample collection period to acclimate the cows to the experimental conditions and feed. This research has received approval for animal husbandry and scientific research from Institutional Animal Care and Use Committee, Rajamangala University of Technology Srivijaya (ID# IAC 04-12-63).

2.2 Ruminal fluid collection

In the final day of the experiment, rumen fluid samples were collected from each experimental group before (0 hours) and after feeding (4 hours). Using aseptic techniques, rumenocentesis was performed with an 18 G, 4-inch needle inserted through the left abdominal wall. The needle was advanced 20 centimeters above the pelvic brim to collect 25-30 milliliters of rumen fluid (Duffield *et al*., 2004), which was immediately measured for pH using a tabletop pH meter (HORIBA, Kyoto, Japan). The collected fluid was then divided: 1) One milliliter was mixed with 9 milliliters of 10% formalin solution for preserving rumen protozoa population analysis and 2) Five milliliters were supplemented with 5 milliliters of 1 M H_2SO_4 for volatile fatty acids (VFAs) analysis, specifically acetate (C2), propionate (C3), and butyrate (C4).

Table 1 The chemical composition of the basal diets, including both concentrate and roughage, utilized in the experiment was analyzed on a dry matter basis.

Composition	Concentrate	Roughage
Crude protein (%)	15.71	5.48
Fat (%)	4.74	0.78
Ash (%)	9.94	5.79
Gross Energy (cal/g)	4,306.55	3,942.63

2.3 Evaluation of Protozoa Motility Scores

Rumen fluid was collected and immediately transferred to a sterile glass slide. A coverslip was applied, and protozoal motility was observed under a 40x magnification microscope. Protozoal motility scores were assessed using light microscopy according to the methodology described by Shah *et al*. (2018). Protozoa in the rumen were categorized into four motion levels based on their observed characteristics under the microscope. Level 3 indicates rapid movement and densely packed group, Level 2 represents moderately fast movement and moderately packed group, Level 1 signifies slow movement and small groups, while Level 0 denotes no movement and dispersed protozoa with a low number of living organisms.

2.4 Counting and Classifying the Population of Rumen Ciliated Protozoa

Ciliated protozoa in the rumen were examined by diluting samples with 10% formalin, using Lugol's iodine staining, and counting under a microscope. Protozoan types based on genus were identified using a microscopic method described by Ogimoto and Imai, (1981) and Dehority (2018). Protozoa counting was conducted by considering the genus types per milliliter.

2.5 Analysis of Volatile Fatty Acids in Rumen Fluid

The analysis of volatile fatty acids (VFAs) in rumen fluid, which are crucial components, including acetate (C2), propionate (C3), and butyrate (C4), was conducted using High-Performance Liquid Chromatography (HPLC; Model LC-20A, Shimadzu, Kyoto, Japan). The HPLC system utilized a C18 column at a temperature of 30 degrees Celsius. The mobile phase consisted of 10mM potassium dihydrogen phosphate (KH_2PO_4) with a flow rate of 1.5 milliliters per minute, and detection was performed at a wavelength of 210 nanometers. The injection volume for the sample was 20 microliters, and the analysis time was set at 20 minutes.

2.6 Statistical Analysis

In this study, statistical analysis was conducted using the software IBM® SPSS® Statistics version 20. The statistical tests employed for hypothesis testing included One-Way Analysis of Variance (ANOVA) to assess variance and post-hoc tests, specifically Dunnett's method, to compare mean differences between experimental and control groups. The statistical results will be presented as mean values with the corresponding Standard Error of the Mean (SEM). Furthermore, the significance level for all statistical tests will be established at a 95% confidence level ($P < 0.05$) and a 99% confidence level ($P < 0.01$).

3. Results and Discussion

The impact of yeast supplementation on the ruminal pH

Our results demonstrated that the SC2 group exhibited a ruminal pH of 6.91 ± 0.02, which was significantly different from the control group (7.16 ± 0.66) and the SC4 group (7.39 ± 0.07) before feeding ($P < 0.05$). However, no significant differences were observed in ruminal pH at 4 hours post-feeding among the three experimental groups. Postprandial ruminal pH measurements showed the SC2 group at 6.40 ± 0.16, the SC4 group at 6.51 ± 0.06, and the control group at 6.68 ± 0.03, as illustrated in Figure 1. These findings are consistent with previous studies reporting an increase in ruminal pH following yeast supplementation in sheep and dairy cows (Thrune *et al*., 2009; Silberberg *et al*., 2013; Diaz *et al*., 2018). The consistently low ruminal pH in the SC2 group suggested either enhanced microbial acidogenesis or reduced buffering capacity. Potential mechanisms include alterations in rumen microbiota (Kim *et al*., 2018), increased fermentation (Li *et al*., 2021) and decreased saliva production (Dijkstra *et al*., 2012). In addition, previous studies suggested that the effect of SC supplementation on ruminal pH may vary due to yeast dosage and/or the composition of dietary oncentrates, which influence rumen digestibility (Bach *et al*., 2007; Thrune *et al*., 2009). Further investigation should involve characterizing the rumen microbiome within each group and assessing saliva flow rates to elucidate the factors contributing to the observed differences in ruminal pH.

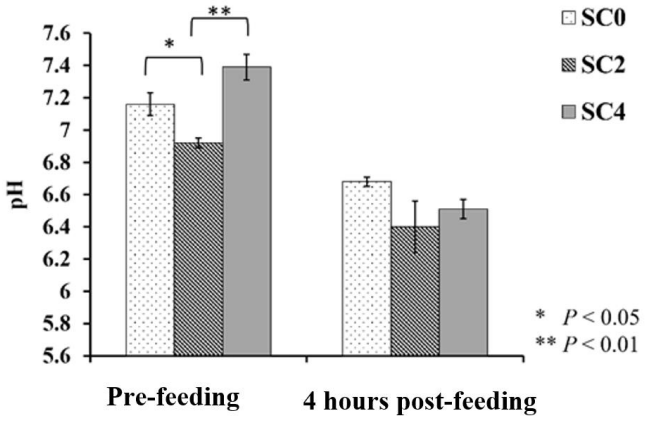


Figure 1 Ruminal pH levels of lactating beef cows that received different quantities of yeast powder at pre-feeding and 4-hours post-feeding on the 21st day of the experiment. The symbols * and ** denote significant differences with *P*-values of < 0.05 and < 0.01, respectively.

Table 2 Comparative Analysis of Rumen Protozoa Motility Scores Pre-feeding and 4 Hours Post-Feeding in Various Yeast-Supplemented Groups.

Time post-feeding	Protozoa Motility Score			*P*-value
	SC0	SC2	SC4	
0 hours	3.00 ± 0.00[a]	2.33 ± 0.33[a]	1.00 ± 0.57[b]	0.027
4 hours	2.00 ± 1.00	3.00 ± 0.00	2.66 ± 0.33	0.533

a vs b: a significant difference in same row ($P < 0.05$)

The impact of yeast supplementation on the rumen ciliated protozoa

Our results showed that pre-feeding, the movement of ciliated protozoa in the rumen fluid differed significantly among the control group, SC2, and SC4, with scores of 3.00 ± 0.00, 2.33 ± 0.33, and 1.00 ± 0.57, respectively (Table 2). The SC4 group exhibited significantly slower protozoa motility compared to the control group ($P < 0.05$). However, no statistically significant differences in protozoal motility were observed among the groups four hours post-feeding. Mean motility scores were 3.00 ± 0.00 for SC2, 2.66 ± 0.33 for SC4, and 2.00 ± 1.00 for the control group (Table 2). These findings suggest a potential antiprotozoal effect of the higher yeast supplementation level. However, this effect was not sustained post-feeding, as no significant differences in protozoal motility were observed among groups. The transient nature of this inhibition may be attributed to the complex dynamics of the rumen environment, including fluctuations in pH and substrate availability. The observed decrease in protozoal motility is likely due to an elevated pre-feeding ruminal pH. Ciliated protozoa are known to be sensitive to pH extremes (Clarke, 1977; Dehority, 2005; Franzolin and Dehority, 2010), suggesting that the strongly acidic or alkaline environment indirectly inhibited their movement.

Table 3 Average Number of Rumen Protozoa Genera per Milliliter (x10^5 cells) in Various Yeast-Supplemented Groups Pre-feeding and 4 Hours Post-Feeding.

Time post-feeding	Protozoa genera	Number of Protozoa (x10^5 cells/mL)			*P*-value
		SC0	SC2	SC4	
0 h	*Entodinium spp.*	8.5 ± 0.51	17.80 ± 5.10	5.87 ± 0.72	0.067
	Diplodinium spp.	0.92 ± 0.92	1.75 ± 0.88	0.00 ± 0.00	0.312
	Polyplaston spp.	0.69 ± 0.69	0.04 ± 0.04	0.04 ± 0.04	0.469
	Eudipladinium spp.	0.87 ± 0.62	0.32 ± 0.20	0.83 ± 0.36	0.627
	Enodinium spp.	0.32 ± 0.16	0.13 ± 0.09	0.00 ± 0.00	0.182
	Metadinium spp.	0.27 ± 0.27	0.18 ± 0.09	0.00 ± 0.00	0.533
	Diploplastron spp.	0.64 ± 0.64	0.27 ± 0.21	0.00 ± 0.00	0.541
	Eremoplastron spp.	0.87 ± 0.20	1.61 ± 0.44	0.87 ± 0.43	0.346
	Chronina spp.	1.38 ± 0.57	2.08 ± 0.24	1.29 ± 0.56	0.500
	Dasytricha spp.	12.11 ± 7.33	5.04 ± 0.24	4.76 ± 2.06	0.465
	Isotricha spp.	4.57 ± 2.91	1.57 ± 0.46	3.88 ± 2.77	0.656
	Total	31.25 ± 12.57	67.64 ± 14.61	30.79 ± 6.96	0.509
4 h	*Entodinium spp.*	13.40 ± 4.36	16.27 ± 4.52	10.81 ± 5.68	0.744
	Diplodinium spp.	2.17 ± 2.17	2.91 ± 1.24	1.15 ± 1.15	0.747
	Polyplaston spp.	1.06 ± 0.86	0.00 ± 0.00	0.23 ± 0.23	0.370
	Eudipladinium spp.	0.88 ± 0.88	1.06 ± 0.46	0.46 ± 0.33	0.782
	Enodinium spp.	0.00 ± 0.00	0.18 ± 0.18	0.00 ± 0.00	0.422
	Diploplastron spp.	0.00 ± 0.00	0.09 ± 0.09	0.00 ± 0.00	0.422
	Eremoplastron spp.	1.66 ± 0.42	1.98 ± 0.40	1.43 ± 0.36	0.638
	Chronina spp.	1.61 ± 0.53	1.66 ± 1.24	4.57 ± 2.95	0.486
	Dasytricha spp.	32.50 ± 14.28	50.90 ± 6.08	41.93 ± 6.64	0.459
	Isotricha spp.	12.66 ± 6.93	13.17 ± 4.40	7.03 ± 2.56	0.645
	Total	67.56 ± 5.72	88.26 ± 20.89	65.97 ± 28.51	0.690

The research findings revealed that there were 1 1 genera: *Entodinium, Diplodinium, Polyplaston, Eudipladinium, Enodinium, Metadinium, Diploplastron, Eremoplastron, Chronina, Dasytricha, and Isotricha* during the study period (Table 3). These ciliated protozoa significantly contribute to the breakdown of feedstuffs, particularly complex carbohydrates, proteins, and lipids, with each species possessing unique functional capabilities (Choudhury *et al.*, 2015; Newbold *et al.*, 2015). Our investigations reveal that SC supplementation had no discernible impact on the quantities of ruminal protozoa at the genus level across the two examination periods ($P > 0.05$). Moreover, while SC supplementation did not significantly affect the overall abundance of ruminal protozoa at the genus level, the SC2 group consistently demonstrated higher total protozoal populations both pre- and post-feeding. Prior to feeding, a noticeable tendency toward an increase in the number of *Entodinium* spp. in the rumen of SC2 cows was observed compared to the other groups ($P = 0.067$). These species are key for starch and plant cell wall degradation, contributing significantly to VFA production (Wang *et al.*, 2019). These findings suggest that SC supplementation might be influencing the protozoal community structure rather than simply increasing the total number of organisms. In contrast to the study by Kowalik *et al*. (2012), which observed increased rumen protozoa, including *Entodinium*, in Jersey heifers fed

yeast metabolites, the absence of a similar response to live SC supplementation highlights the complexity of yeast product effects on ruminal protozoa. Furthermore, variations in yeast product composition and dosage across studies hinder direct comparisons and limit definitive conclusions. The observed increase in *Entodinium* spp. within the SC2 group warrants further investigation. These results align with Galip (2006), who reported increased rumen ciliate protozoa populations following dry yeast supplementation in rams. However, contrasting results have been reported. Phesatcha *et al*. (2021) observed decreased rumen protozoa and methanogen populations in Thai native beef cattle following dry yeast supplementation. Concurrently, increased abundances of cellulolytic bacteria, including *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens*, and *Fibrobacter succinogenes*, were reported (Zhu *et al.*, 2017; Phesatcha *et al.*, 2021). Our findings suggest that SC supplementation had a minimal effect on rumen ciliated protozoa populations, potentially influencing feed degradation and VFA production. The complex interactions within the rumen microbiome, including dynamic fluctuations in pH, likely play a significant role in determining the response to yeast supplementation. To gain a deeper understanding of these mechanisms, future research should focus on quantifying the relative abundances of total bacteria, fiber-degrading bacteria, and methanogens. This will help elucidate how SC supplementation interacts with the broader rumen microbial community and its impact on key metabolic processes.

The impact of yeast supplementation on the ruminal VFAs production

The results of the ruminal VFA concentrations, as shown in Table 4. Pre-feeding, the SC4 group exhibited significantly higher total VFA concentrations (179.03 ± 1.43 mmol/L) compared to the control and SC2 groups ($P < 0.05$). Acetate (A) concentrations tended to be higher in the SC4 group (103.65 ± 2.40 mmol/L) compared to the other groups ($P = 0.057$), although this difference did not reach statistical significance. No significant differences were observed in propionate (P), butyrate (B), A:P, or A+B:P ratios among the groups. Post-feeding, the SC2 group displayed the highest total VFA production (280.60 ± 16.86 mmol/L; $P < 0.05$), with both the SC2 and SC4 groups exhibiting significantly elevated acetate concentrations compared to the control group ($P < 0.05$). However, propionate and butyrate levels did not differ significantly among the groups. The SC2 group demonstrated significantly higher A:P and A+B:P ratios compared to the other groups ($P < 0.05$).

The observed pre-feeding elevation in total VFA concentration, particularly acetate, within the SC4 group suggests a potential stimulation of microbial fermentation by the higher yeast supplementation level. This finding is in agreement with previous studies demonstrating increased VFA production following yeast supplementation (Marden *et al.*, 2008). Conversely, Phesatcha *et al.* (2021) reported an increase in total VFAs and propionic acid, accompanied by a decrease in acetic and butyric acid levels, when beef cattle were fed urea-lime-treated rice straw supplemented with 3 g/day of dry yeast. These changes were attributed to the stimulation of cellulolytic bacterial populations. However, the lack of significant differences in other VFA parameters indicates that the influence of yeast on VFA profile may be complex and dependent on dietary factors and experimental conditions.

The post-feeding increases in total VFAs and acetate in both the SC2 and SC4 groups suggests a sustained stimulatory effect of yeast on rumen fermentation. The higher A:P and A+B:P ratios observed in the SC2 group may indicate shifts in microbial populations toward a more fibrolytic profile, leading to increased acetate production. These results are similar to previous research demonstrating that live yeast supplementation can augment fiber digestion in dairy cows fed high-forage diets by stimulating fibrolytic bacteria and fungi, thereby facilitating microbial colonization of fibrous substrates (Chaucheyras‐Durand *et al.*, 2016). The lack of significant differences in propionate and butyrate levels suggests that the primary effect of dry yeast supplementation on VFA production is an increase in acetate, potentially through the stimulation of cellulolytic bacteria. However, further research is required to elucidate the specific mechanisms underlying these changes and to determine the long-term implications for animal performance.

Table 4 The concentration (mmol/L) of three major ruminal VFAs (Acetate, Propionate and Butyrate) in the different yeast-supplemented groups, both pre-feeding and 4-hours post-feeding.

Time post-feeding	VFAs (mmol/L)	Treatments			*P*-value
		SC0	SC2	SC4	
0 hours	Total VFAs	158.25±5.01[a]	151.19±8.72[a]	179.03±1.43[b]	0.036
	Acetate (A)	88.59±3.24	89.36±5.36	103.65±2.40	0.057
	Propionate (P)	27.75±2.26	28.28±1.91	31.57±1.84	0.405
	Butyrate (B)	41.90±0.81	33.56±7.39	43.80±0.80	0.278
	A:P ratio	3.22±0.21	3.17±0.15	3.31±0.27	0.893
	A+B:P ratio	4.75±0.30	4.36±0.15	4.71±0.37	0.605
4 hours	Total VFAs	192.57±13.88[a]	280.60±16.86[b]	252.68±17.79[b]	0.022
	Acetate (A)	100.18±7.99[a]	168.08±7.27[b]	143.60±15.07[b]	0.011
	Propionate (P)	46.20±3.00	52.93±2.08	54.54±1.39	0.084
	Butyrate (B)	37.91±8.06	40.55±8.07	53.38±2.20	0.292
	A:P ratio	2.16±0.05[a]	3.18±0.06[b]	2.62±0.22[a]	0.005
	A+B:P ratio	3.16±0.05[a]	4.29±0.11[b]	3.62±0.22[a]	0.004

4. Conclusion

This study investigated the effects of dry yeast supplementation on ruminal pH, ciliated protozoa, and fermentation parameters in lactating beef cows. While yeast supplementation resulted in a transient decrease in ruminal pH, it had minimal effects on protozoa populations and motility. However, a positive impact on rumen fermentation was evident, as indicated by increased total VFA and acetate production. These findings highlight the intricate relationship between yeast, rumen microorganisms, and fermentation processes. Future research is necessary to clarify the specific mechanisms involved and optimize yeast supplementation strategies for improving the digestive efficiency and nutrient utilization of lactating beef cows.

5. Recommendations

The findings of this study diverge from previous research, likely due to the use of high-quality forage. To induce more pronounced treatment effects in future investigations, it is recommended to employ a lower-quality diet to create a more challenging environment for the animals. Moreover, the study did not identify significant benefits from additional yeast application, suggesting potential methodological limitations. Future research should refine the approach, with a particular focus on yeast-related factors, to enhance the study's validity and generate more meaningful insights. The relatively small sample size per group may have constrained statistical power, emphasizing the need for larger sample sizes. Alternatively, a Latin square design could help minimize within-group variation, leading to more reliable results.

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