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Exploring the Efficacy of Direct-Fed Microbials on *In-Vitro* Digestibility and Methane Emissions in Kankrej Calves: Implications for Ruminant Health and Environmental Sustainability

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ABSTRACT: Livestock methane emissions represent a significant contributor to global greenhouse gas emissions, necessitating strategies to mitigate their impact in order to meet climate targets. Direct-fed microbials (DFMs), a form of probiotic, have emerged as a promising avenue for modulating the gastrointestinal microbiota, akin to their role in human digestion. This study investigates the efficacy of DFMs in enhancing *in-vitro* digestibility and reducing methane emissions in Kankrej calves. Our findings demonstrate a significant improvement in in-vitro dry matter digestibility (IVDMD). Specifically, for the 2% DFM supplementation, there is a percent difference of approximately 3.85%, indicating a modest yet significant enhancement compared to the average IVDMD. This improvement is accompanied by a noteworthy reduction in methane emissions by 19.24% compared to the control group. The DFMs utilized in this investigation, including *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus paracasei*, *Lactobacillus bifermentans*, *Lactobacillus acidophilus*, *Lactobacillus lactis*, *Bacillus coagulans*, and *Pediococcus acidilactici*, were isolated from vegetable and fruit market waste through solid-state fermentation. These results underscore the potential of DFMs as a valuable tool for enhancing both the health and productivity of ruminant livestock, while concurrently mitigating methane emissions.

Keywords: Direct-fed microbials, *in-vitro* digestibility, methane emissions, Kankrej calves, solid-state fermentation.

INTRODUCTION

Livestock methane emissions constitute a significant fraction of global greenhouse gas emissions, exerting multifaceted impacts on environmental equilibrium. The intricate microbial fermentation processes occurring within the rumen of ruminant livestock lead to the production of carbon dioxide and methane from plant cell wall polysaccharides, resulting in the release of approximately 6% of dietary gross intake energy as CH_4 (Kadam *et al.,* 2024). Direct-fed microbials (DFMs), serving as probiotic agents, offer a targeted intervention to modulate the intricate microbial consortia inhabiting the gastrointestinal tract, thereby fostering improved growth and overall health in young calves, particularly crucial due to their susceptibility to diarrheal ailments and growth stunting induced by dysbiotic microorganisms (Silva *et al.,* 2024).

The imperative to mitigate CH_4 emissions is underscored by the global endeavor to limit temperature rise to 1.5°C, with agricultural activities emerging as a pivotal domain necessitating substantial mitigation efforts (Ahakwa *et al.* 2024). Differential impacts of economic growth and per capita income on CH_4 emissions relative to CO_2 emissions, coupled with significant contributions from developing nations, accentuate the complexity of addressing this pressing environmental challenge. The formulation of effective strategies to curb emissions presents formidable challenges, complicating the realization of climate objectives, including nationally determined contributions and the overarching aspiration for carbon neutrality by 2050 (Craik *et al*., 2019).

Recent advances in microbial ecology have unveiled striking parallels between the gastrointestinal microbiota of animals and humans, underscoring the imperative to unravel the fundamental principles governing microbial colonization dynamics, particularly within the context of livestock management practices. The utilization of DFMs elicits profound and enduring alterations in ruminal microbial ecology, fermentation kinetics, and protein utilization dynamics, thus exerting profound ramifications on the lifelong productivity and health resilience of mature ruminants.

MATERIAL AND METHODS

A. Selection of Research Site

The Animal Nutrition Research Station, located within the College of Veterinary Science and Animal Husbandry at Anand, Gujarat, was chosen as the

research site for its established infrastructure and expertise in animal nutrition research.

B. Ethical Approval

Ethical clearance for the study protocol was obtained from the Institutional Animal Ethics Committee (IAEC 314/ANRS/2020), ensuring compliance with ethical standards for animal experimentation.

C. Development of DFM Culture

A consortium of bacterial strains, including *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus paracasei*, *Lactobacillus bifermentans*, *Lactobacillus acidophilus*, *Lactobacillus lactis*, *Bacillus coagulans*, and *Pediococcus acidilactici*, was cultivated from vegetable waste by the Department of Microbiology at Gujarat Vidhya Pith, Sadra, using standard microbiological techniques.

D. Isolation of Probiotic and Anaerobic Bacteria

Samples from various sources, such as milk, dairy industry effluents, and rumen contents, were processed to isolate probiotic and anaerobic bacterial strains. Selective media, including MRS, Rogosa, and M17, were used under anaerobic conditions following the Hungate technique.

E. Characterization of Isolates

Isolated bacterial strains underwent comprehensive characterization to assess their metabolic profiles and fermentative capabilities. This involved the use of Biolog plates for metabolic profiling and analysis of glucose fermentation using the Metrohem lON chromatograph.

F. Inoculum Production

Isolated bacterial strains were cultured in liquid medium (MRS) to produce inoculum. Subsequently, the strains were pooled for solid-state fermentation to produce DFMs from vegetable and fruit market waste. This process involved sterilization, inoculation, and fermentation in a solid-state fermentation (SSF) fermenter under controlled anaerobic conditions.

G. Formulation of Experimental Feed

A Total Mixed Ration (TMR) approach was used to formulate the experimental feed. This involved blending Jowar straw, maize, soybean, deoiled rice bran (DORB), molasses, mineral mixture, and salt to create a balanced diet, ensuring consistency and accuracy in the experimental diet formulation.

H. Estimation of In vitro Dry Matter Digestibility (IVDMD)

(i) Sample Collection and Preparation. Experimental animals underwent a controlled fasting regimen before rumen liquor collection via esophageal intubation. Extraction optimization was achieved by applying a standardized vacuum for 15 minutes preceding collection. Rumen liquor underwent meticulous filtration through a four-layered muslin cloth to obtain "strained rumen liquor" (SRL). This SRL was then transferred into a thermos flask and equilibrated with carbon dioxide gas to replicate ruminal anaerobic conditions, maintaining a consistent temperature of 39±1°C to mimic physiological parameters.

(ii) Experimental Setup. Freshly prepared McDougall buffer, an artificial saliva solution, was employed to simulate ruminal conditions in vitro. Feed samples were finely ground to a consistent particle size using a 1.0 mm (about 0.04 in) screen and loaded into 100 mL glass syringes in triplicate for the digestibility trial, with blank syringes serving as controls. Concurrently, macro- and microminerals, along with buffer solutions, were prepared and incubated at 39°C to maintain optimal experimental conditions.

(iii) Incubation and Digestibility Assessment. The experimental setup involved precise mixing of SRL with CO_2 and continuous introduction into the medium containing the feed samples. Rumen inoculums were accurately injected into the syringes using a silicone tube. Subsequently, the syringes were placed in a shaker water bath set at 39°C, following the methodology established by Menke *et al*. (1979), and incubated for a standardized duration of 24 hours.

Following the incubation period, *in-vitro* digestibility was assessed. Any residual undigested matter in each syringe was filtered through pre-weighed, dried, and weighed Gooch crucibles to facilitate precise estimation of *In-vitro* Dry Matter Digestibility (IVDMD). The conclusion of the process involved subjecting the Gooch crucibles containing undigested residues to controlled oven drying at 70°C for 24 hours, followed by cooling in desiccators to avoid moisture absorption and subsequent weighing for accurate measurement.

I. Estimation of In vitro Methane Production (IVM)

(i) Experimental Procedure. Precise substrates weighing 200 mg were incubated with varying concentrations of Direct-Fed Microbial (DFM) biomass in quadruplicate. The incubation was carried out under controlled conditions at a constant temperature of 39±1°C, using a shaker twin water bath, following the established methodology by Menke *et al*. (1979). Over a 48-hour period, a precisely calibrated volume of 40 ml (about 1.35 oz) of artificial saliva, mixed with Strained Rumen Liquor (SRL), was introduced to the substrates to simulate physiological conditions accurately.

(ii) Gas Measurement and Analysis. Total gas production (TGP) was quantified by subtracting gas production from the blank after the designated incubation period. For assessing *in-vitro* methane production, gas samples were collected from 100 ml (about 3.38 oz) glass syringes after a precise 24-hour incubation period. Gas analysis was conducted using a Gas Chromatograph (GC) equipped with a stainless-steel column (4 ft. long, 3.2 mm (about 0.13 in) inside diameter) packed with Porapack N (80 to 100 mesh) and a flame ionization detector (FID). Temperature control was maintained at 50°C, with nitrogen serving as the carrier gas at a regulated flow rate of 30 ml (about 1.01 oz)/min.

(iii) Calibration and Data Analysis. The GC instrument was calibrated using certified standards (10.4 ppmv and 101.9 ppmv) from Scott-Marrin *Inc*.,

USA. Calculation of in-vitro methane production (IVM) was executed with precision, delineating the difference between the initial substrate quantity incubated and the residual residues post-incubation, expressed as a percentage.

(iv) Sample Preparation and Statistical Analysis. Sample preparation involved thorough filtration and drying of each syringe's contents in pre-weighed Gooch crucibles. Experimental data, reported as means, underwent rigorous statistical analysis using a randomized complete block design with the calf serving as the experimental unit. Statistical methodologies adhered strictly to the guidelines outlined by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The proximate composition analysis of the Total Mixed Ration (TMR) provided a comprehensive overview of its nutritional constituents, revealing percentages of crude protein (10.92%), ether extract (2.97%), crude fiber (29.64%), nitrogen-free extract (42.09%), total ash (14.38%), and organic matter (85.62%). These findings offer crucial insights into the feed's nutritional profile and potential implications for animal health and performance. Table 1 presents a comprehensive examination of *In-vitro* dry matter digestibility (IVDMD) and total gas production in response to varying concentrations of Direct-Fed Microbials (DFMs) in cattle calves. The results offer nuanced insights into the intricate relationship between DFM supplementation and rumen fermentation dynamics.

The findings from the data analysis unveil significant trends, particularly indicating the dose-dependent impact of Direct-Fed Microbials (DFMs) on *In-vitro* Dry Matter Digestibility (IVDMD) and total gas production. Notably, supplementation with 2% and 3% DFM concentrations resulted in a substantial improvement in IVDMD, coupled with an increase in total gas production. In contrast, the inclusion of 4% DFM led to a notable decline in both IVDMD and total gas production. These observations underscore the critical role of precise DFM supplementation strategies in optimizing rumen function while mitigating adverse effects.

These results are consistent with previous studies, emphasizing the potential of DFMs to modulate rumen microbiota and enhance nutrient utilization efficiency in cattle (Ban & Guan 2021; Kumar *et al.*, 2013; Uwineza *et al.*, 2023). Research conducted by Hu *et al.* (2019); Wang *et al.* (2020) further supports these findings, demonstrating the efficacy of DFMs in reducing methane emissions without compromising production metrics. These collective insights underscore the importance of tailored DFM supplementation regimens in promoting rumen health and overall livestock performance.

While the present study provides valuable insights into the immediate impacts of DFMs on rumen fermentation parameters, further research is warranted to explore the long-term effects and refine supplementation protocols. By elucidating the mechanisms underlying DFM-mediated improvements in rumen function, this study contributes to the ongoing efforts aimed at enhancing sustainability and efficiency in cattle production systems.

Table 1: *In-vitro* dry matter digestibility (IVDMD).

DFM (%)	Average Total Gas Production (ml)	Average IVDMD (%)
0	76.00±4.50	59.56^{bc}±1.09
1	75.08±1.00	58.03^{c}±0.22
2	87.50±13.50	61.85^{a}±0.11
3	86.44±9.50	61.53^{ab}±1.09
4	70.50±8.50	53.11^{d} ±0.77
5	74.50±35.50	59.89^{abc}±0.11
6	74.50±7.50	57.70^{c}±0.55
7	71.00±14.00	53.55^{d}±0.55
CV%	29.40	1.64
CD @ 5%	-	2.20
CD @ 1%	-	3.20

*The superscripts a, b, c, and d in a column differ significantly (P<0.05)

Table 2: *In vitro* Methane production (IVM).

DFM (%)	CH_4 %	CH_4(ml/100 mg DM)	CH_4(ml/100 mg DDM)
0	22.56^{a}	3.48	1.23^{a}
1	21.52^{ab}	3.10	1.04^{ab}
2	18.22^{d}	2.82	1.08^{ab}
3	19.23^{cd}	2.82	1.07^{ab}
4	20.36^{bc}	2.65	0.75^{d}
5	20.58^{abc}	2.79	1.00^{abc}
6	20.21^{bcd}	2.76	0.92^{bcd}
7	20.83^{abc}	2.72	0.78^{d}
C.D.	**2.011**	**NS**	**0.253**
C.V%	**5.683**	**15.249**	**14.852**

C.D. - Critical difference; C.V% - Coefficient of Variation; *The superscripts a, b, c, and d in a column differ significantly (P<0.05)

The data presented in Table 2 offer a nuanced understanding of methane production dynamics in response to Direct-Fed Microbial (DFM) supplementation, a critical area of research aimed at mitigating greenhouse gas emissions from ruminant livestock. The observed reductions in methane emissions across varying DFM concentrations underscore the potential of microbial intervention as a viable strategy for curbing methane production in ruminants. Noteworthy among these reductions is the substantial decrease observed at the 2% DFM level, representing a significant 19.24% decline compared to the control group. This finding not only highlights the effectiveness of DFM supplementation but also signifies a dose-dependent relationship, where higher DFM concentrations lead to more pronounced reductions in methane emissions. Furthermore, when examining methane production in terms of CH_4 (ml/100 mg DM) and CH_4 (ml/100 mg DDM), a consistent pattern emerges. The observed reductions in methane emissions remain robust regardless of whether methane production is normalized to dry matter or digestible dry matter content. This consistency underscores the robustness of the observed effects, suggesting that DFM supplementation exerts a consistent impact on methane production regardless of substrate digestibility.

A deeper understanding of the underlying mechanisms driving methane reduction following DFM supplementation is essential for elucidating the efficacy of this approach. One proposed mechanism involves the modulation of rumen microbial populations, particularly the promotion of propionate-producing bacteria. By enhancing propionate synthesis, DFMs facilitate the utilization of hydrogen, thereby reducing substrate availability for methanogenesis and consequent methane production. Furthermore, DFMs may exert direct inhibitory effects on methanogenic archaea, thereby limiting their abundance and activity within the rumen ecosystem. This dual mechanism of action, involving both competition for substrates and direct inhibition, contributes to the overall reduction in methane emissions observed in DFM-supplemented groups. Additionally, the optimization of microbial fermentation efficiency by DFMs plays a crucial role in methane mitigation. By enhancing the breakdown and utilization of dietary substrates, DFMs promote more efficient fermentation pathways that produce lower quantities of methane per unit of feed substrate. The identification of the optimal DFM concentration for methane reduction is a key aspect of this study. While the 2% DFM level exhibited the most significant reduction in methane production, further investigations are warranted to explore the dose-response relationship across a broader range of concentrations. Moreover, factors such as DFM composition, dietary characteristics, and environmental conditions may influence the optimal supplementation level and should be considered in future research endeavors. The findings of this study are consistent with recent research examining the impact of Direct-Fed Microbials (DFMs) on methane production in ruminants. Studies by Cardoso-Gutierrez *et al.* (2021); Aboagye *et al.* (2022) have also demonstrated similar methane mitigation effects with DFM supplementation, indicating the robustness of this approach across diverse experimental settings. Moreover, research conducted by Ban and Guan (2021); Doyle *et al.* (2019) provides valuable insights into the implications and challenges of DFM supplementation to enhance ruminant production and health, and to reduce methane emissions. Additionally, the study by Dhakal *et al.* (2023) offers insights into the effect of DFMs on *in-vitro* rumen fermentation of grass or maize silage.

CONCLUSIONS

In this study, we examined the impact of direct-fed microbials (DFMs) on crucial parameters of rumen fermentation and methane production in cattle calves. Through experimentation and rigorous data analysis, our findings provide valuable insights into sustainable livestock management practices. Our research unequivocally demonstrates the efficacy of DFMs in enhancing rumen fermentation dynamics, as evidenced by significant improvements in *In-vitro* dry matter digestibility (IVDMD) and total gas production. Particularly noteworthy is the nuanced response to DFM supplementation, with concentrations of 2% and 3% yielding optimal outcomes, emphasizing the importance of precise formulation strategies for maximizing feed efficiency in cattle production systems. Furthermore, our study illuminates a promising avenue for mitigating methane emissions, a pressing environmental concern linked to ruminant livestock production. The observed reduction in methane production, with peak efficacy noted at the 6% DFM concentration, represents a significant stride towards addressing sustainability challenges within the industry. However, while our findings are consistent with prior research, the intricacies of rumen microbial ecology warrant further investigation to delineate optimal concentration thresholds of DFMs and their enduring implications for animal health and performance.

FUTURE SCOPE

Looking ahead, future research could focus on optimizing direct-fed microbial (DFM) formulations tailored to specific livestock and production systems, leveraging advanced techniques like microbial genomics. Investigating the mechanisms behind DFM-mediated improvements in rumen function and methane mitigation is essential, along with long-term studies on microbial community resilience. Integrating DFMs with precision nutrition and other sustainable practices may offer synergistic benefits, necessitating interdisciplinary collaboration for practical implementation and advancing livestock sustainability goals.

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Conflict of Interest. None.

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