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SEQUENTIAL CULTURE OF RUMEN FLUID AS A SUSTAINABLE INOCULANT FOR *IN VITRO* RUMINANTS FEED EVALUATION

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ABSTRACT: Rumen fluid plays a crucial role in *in vitro* studies for evaluating ruminant feed. Maintaining microbial activity in rumen fluid can serve as a breakthrough approach to reducing dependence on fresh rumen fluid collection by utilizing sequential culture techniques. This study aimed to assess the effectiveness of rumen microbial inoculants through sequential cultures with a 48-hour incubation period. A completely randomized design was applied with four treatments: K1 = Culture 1 (inoculant derived from fresh rumen fluid), K2 = Culture 2 (inoculant derived from Culture 1), K3 = Culture 3 (inoculant derived from Culture 2), and K4 = Culture 4 (inoculant derived from Culture 3). The test substrates included dwarf elephant grass and *Indigofera zollingeriana* leaves using analysis *in vitro* sequential cultures adapted from Tilley and Terry (1963) and the Consecutive Batch Culture (CBC) method. Parameters measured included rumen fermentation characteristics such as pH, ammonia nitrogen (N-NH_3) concentration, total volatile fatty acid (VFA) production, and dry matter digestibility. Data were analyzed using analysis of variance (ANOVA) followed by Tukey's HSD (Honest Significant Difference) test. The results showed that the sequential culture process significantly affected *in vitro* rumen fermentation characteristics. The pH remained stable within the optimal range (6.67–6.78). Increased culture sequences enhanced N-NH_3 concentration, total VFA production, and dry matter digestibility. It can be concluded that rumen microbial inoculants remain effective up to the fourth sequential culture for *in vitro* evaluation of ruminant feeds.

Keywords: Digestibility, Dry matte, Inoculant, Microbial viability, Sequential culture

INTRODUCTION

Ruminants possess a complex gastric system comprising four compartments: the rumen, reticulum, omasum, and abomasum, with the rumen being the largest and most functionally significant (Palma-Hidalgo et al., 2021). The rumen contains a heterogeneous mixture of feed, water, fermentation by-products, and a dense population of living microorganisms. The rumen microbiota is diverse and dynamic, influenced by geographical region and the type of feed consumed (Silva et al., 2024). The primary microbial groups present in the rumen include bacteria, protozoa, and fungi (Castillo and Hernández, 2021). The adaptability and structural complexity of these microbial communities enable them to play a critical role in breaking down plant biomass into microbial protein, volatile fatty acids (VFAs), and other fermentation end-products that serve as essential nutrients for the host animal's metabolism (Ji et al., 2017).

The *in vitro* method for feed evaluation offers several advantages over *in vivo* techniques. It is cost-effective, time-efficient, and allows greater control over incubation conditions (Getachew et al., 2002). *In vitro* methods have been widely adopted in animal nutrition research as preliminary tools before conducting *in vivo* trials, significantly reducing the reliance on experimental animals and overall research costs (Vinyard and Faciola, 2022). The use of rumen fluid microbes in *in vitro* fermentation systems is essential for simulating rumen fermentation dynamics and estimating feed digestibility with results that closely reflect *in vivo* conditions (Raffrenato et al., 2021).

Despite the critical role of rumen fluid in *in vitro* studies, its acquisition poses several challenges. Lodge-Ivey et al. (2009) noted that obtaining rumen fluid typically involves rumen cannulation, which requires surgically fistulated animals. Alternative methods, such as using esophageal or oral cannulae, are less invasive but can stress the animals and risk contamination with saliva (Fortina et al., 2022). Additionally, ethical considerations arise when using live animals as rumen fluid donors (Spanghero et al., 2019). Logistical constraints, including limited availability of donor animals, long-distance transportation, and timing issues, especially when rumen fluid is sourced from slaughterhouses further complicate its use in routine research.

One promising solution is to culture rumen fluid in laboratory settings while maintaining its microbial viability, thus minimizing dependence on cannulated animals, rumenocentesis, or slaughterhouse sources (Tunkala et al., 2022). Creating optimal conditions for the growth of anaerobic rumen microbes requires controlling key environmental factors such as temperature, pH, buffering capacity, osmotic pressure, and redox potential (Castillo-González et al., 2014).

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Maintaining an active culture of rumen fluid over multiple incubation cycles allows researchers to preserve microbial activity for successive *in vitro* degradation assays of various feed types.

The Tilley and Terry (1963) method is a widely used two-stage *in vitro* digestibility assay involving incubation with rumen fluid followed by enzymatic digestion using HCl-pepsin (Zewdie, 2019). This method has demonstrated high correlation with *in vivo* digestibility and remains a standard technique for evaluating feed quality (Tassone et al., 2020). In parallel, the Consecutive Batch Culture (CBC) method, developed by Gascoyne and Theodorou (1988), mimics the rumen environment through sequential inoculation and incubation of subcultures in fresh buffer under controlled conditions. In this system, microbial communities are transferred to new culture media at defined intervals to maintain active fermentation (Mbiriri et al., 2016).

Integrating the 48-hour rumen incubation phase from the Tilley and Terry method with the principles of the CBC system results in a sequential culture technique. This approach aims to produce stable and reproducible rumen fluid inoculants for *in vitro* testing. Rumen fluid collected from donor animals is cultured under controlled laboratory conditions designed to replicate *in vivo* rumen fermentation. Sequential culturing presents a viable alternative to conventional sourcing of rumen fluid, enabling researchers to maintain microbial stability while customizing nutrient and environmental parameters. Therefore, this study was conducted to evaluate the effectiveness of rumen fluid as an inoculant through sequential culturing, by assessing its impact on fermentation characteristics and *in vitro* dry matter digestibility of selected ruminant feedstuffs.

MATERIALS AND METHODS

Ethical considerations

All methodologies and guidelines applied in this experiment were approved by the Animal Ethics Committee for Research and Education at the Faculty of Animal Science, Hasanuddin University, Makassar, prior to the commencement of the study. Ethical approval was granted under the reference number 018/UN4.12/EC/XI/2023, in accordance with the seven WHO ethical standards (2001).

Tool preparation

Feed bags were made from nylon fabric (Depure) measuring 8 × 4 cm with a pore size of 100 µm, based on the method of Carro et al. (1995). A modified U-shaped press was used to form the curved bottom of each bag. To ensure submersion and containment of the feed sample during incubation, each nylon bag was equipped with a 20 g glass weight and secured using a clamp. Prior to use, the bags were dried in an oven at 65°C for 72 hours to remove residual moisture and then weighed to determine their initial dry mass. The lid of the artificial rumen was constructed from a No. 8 rubber stopper, with an upper diameter of 4.5 cm and a lower diameter of 3.8 cm. Two holes (6 mm in diameter) were drilled into the stopper. The first hole was fitted with a 10 cm silicone hose (3 mm inner diameter, 5 mm outer diameter) connected to a gas valve for releasing fermentation gases. The second hole housed a 19 cm silicone hose equipped with a pinch clamp and a 60 mL syringe for transferring the subculture inoculum. The fermentation chamber consisted of a 250 mL polypropylene Erlenmeyer flask with a 4 cm mouth diameter and a height of 13.7 cm. This setup simulated anaerobic rumen fermentation conditions for *in vitro* culture.

Feed sample preparation

The feed ingredients used in this study consisted of a mixture of 70% dwarf elephant grass and 30% *Indigofera zollingeriana*, harvested 70 days after uniform pruning during the dry season. The harvested materials were oven-dried at 70°C for 72 hours until completely dry (Memmert Universal Oven UNB 400). The dried samples were then ground using a 14-mesh grinding machine (B-One DM-120 M) to obtain a uniform particle size suitable for *in vitro* fermentation.

Preparation of artificial saliva

Artificial saliva this solution served as a pH stabilizer and a mineral source during fermentation, providing essential nutrients for sustaining microbial activity in the *in vitro* rumen environment. Artificial saliva, also referred to as McDougall's solution, was prepared according to the formulation described by McDougall, as cited in Close and Karl-Heinz (1986).

Rumen fluid preparation

Rumen fluid was collected from two cattle slaughtered at the CV Akbar Jaya Sejahtera abattoir, located in Tamangapa, Antang, Makassar (slaughter certificate number 06020013030319), Indonesia. Immediately after slaughter, the warm rumen contents were transferred into a thermos box to maintain temperature and preserve microbial viability during transportation to the laboratory. Upon arrival, the rumen solids were filtered using a nylon cloth with 250 µm porosity (Yáñez-Ruiz et al., 2016) to extract the fluid fraction. The resulting rumen fluid was used as the microbial inoculant for the *in vitro* fermentation process.

Experiment design

The stability and fermentative activity of rumen microorganisms after repeated incubation were evaluated using feed samples composed of dwarf elephant grass and *Indigofera zollingeriana* leaves. The assessment employed a sequential culture *in vitro* method adapted from Tilley and Terry (1963) and CBC. This approach was designed to determine the extent to which microbial viability and activity could be maintained across multiple incubation cycles, thus offering a potential alternative to the repeated collection of fresh rumen fluid for use as an inoculant.

The study consisted of four culture stages, where the inoculum from the previous stage was used to initiate the next incubation. The treatment groups were as follows: K1= Culture 1 (inoculant derived from fresh rumen fluid), K2= Culture 2 (inoculant derived from Culture 1), K3= Culture 3 (inoculant derived from Culture 2), K4= Culture 4 (inoculant derived from Culture 3).

Experiment procedure

The first stage of incubation was initiated by weighing 2.5 grams of the feed sample and placing it into a pre-weighed nylon bag. The bag was then equipped with a 20 g glass weight and secured with a clamp to ensure submersion. The bag containing the sample was inserted into an artificial rumen vessel and filled with 250 mL of a 4:1 mixture of freshly prepared rumen fluid and artificial saliva, following the procedure of Tilley and Terry (1963). The vessel was sealed with a ventilated rubber stopper and flushed with CO_2 gas to create anaerobic conditions by displacing residual oxygen.

Incubation was conducted in a Memmert WPE 45 water bath at 39°C for 48 hours, with manual shaking performed twice daily to maintain uniform fermentation. At the end of each incubation, 50 mL of the fermentation medium was withdrawn using a 60 mL syringe and transferred into a new artificial rumen flask containing a fresh 2.5 g feed sample and 200 mL of artificial saliva solution. This subculturing process was repeated across four consecutive culture stages (K1 to K4), each with a 48-hour incubation period under identical conditions. At the end of each culture stage, samples of the remaining inoculant were collected and analyzed to determine pH, ammonia nitrogen (N-NH_3) concentration, total volatile fatty acid (VFA) production, and dry matter digestibility (DMD).

Parameter and laboratory analysis

pH value

The pH of the artificial rumen fluid inoculant was measured immediately after transferring the subculture into the new fermentation medium to assess the stability of the microbial environment. The pH solution analysis of a reference Covington et al. (1985). The electrode was immersed directly into the rumen fluid sample, and the pH value was recorded from the digital display. Following pH measurement, the remaining rumen fluid sample was centrifuged at 10,000 rpm for 15 minutes to separate the supernatant from suspended solids. The resulting supernatant was then stored in a freezer at −20°C for subsequent analysis of ammonia nitrogen (N-NH_3) concentration and total volatile fatty acids (VFA).

N-NH3 concentration

Ammonia nitrogen (N-NH_3) concentration was determined using the Conway microdiffusion method, as described by Thirumalaisamy et al. (2022). To ensure an airtight seal, the rim of the Conway dish was coated with petroleum jelly. One milliliter of the fermentation supernatant was pipetted into one of the outer compartments of the dish, and 1 mL of sodium carbonate (Na_2CO_3) solution was added to the opposite compartment, taking care to avoid premature mixing. A central well in the dish was filled with 1 mL of boric acid (H_3BO_3) solution containing a mixed indicator to absorb the released ammonia. The Conway dish was then sealed and gently tilted to mix the supernatant and Na_2CO_3 solution. The setup was incubated at room temperature (25°C) for 24 hours to allow ammonia gas diffusion into the boric acid. After the diffusion period, the boric acid solution was titrated with 0.0103 N sulfuric acid (H_2SO_4) until a color change from green to red signified the titration endpoint. The volume of H_2SO_4 used was recorded to calculate the N-NH_3 concentration, expressed in millimoles per liter (mM), using the following formula: N-NH_3 (Mm) = Volume of H_2SO_4 x Normality of H_2SO_4 x 1000.

Total volatile fatty acids (VFA)

The total volatile fatty acid (VFA) concentration in the artificial rumen fluid was determined using the steam distillation method, following the procedure outlined by Kromann et al. (1967) and employing a Kjeldahl micro distillation apparatus. This method isolated and quantified the volatile fatty acids produced during microbial fermentation. The procedure began by mixing 5 mL of rumen fluid supernatant with 200 mL of distilled water in a distillation tube. To this mixture, 1 mL of 15% sulfuric acid (H_2SO_4) was added to facilitate the release of volatile fatty acids. The distillate was collected in a receiving Erlenmeyer flask pre-filled with 5 mL of 0.5 N sodium hydroxide (NaOH) and 2–3 drops of phenolphthalein (PP) indicator to maintain alkaline conditions. After the completion of the distillation process, the contents of the Erlenmeyer flask were titrated with 0.25 N hydrochloric acid (HCl) until the color changed from red to colorless, indicating the titration endpoint. The volume of HCl used corresponded to the total VFA concentration in the sample and was expressed in mM, using the following formula: Total VFA = (Vb - Vs) x N-HCl x 1000/5 mM.

Dry matter digestibility

Dry matter digestibility (DMD) of the feed samples was determined following the *in sacco* approach described by Kera et al. (2022), after 48 hours of *in vitro* incubation. Upon completion of incubation, the nylon bags containing the residual feed were carefully removed from the fermentation medium and gently rinsed under running tap water until the rinse water ran clear, indicating the removal of adhering fermentation residues. The washed bags were then oven-dried at a constant temperature of 65°C for 72 hours, or until they reached a stable weight, to ensure complete moisture evaporation. After drying, the bags were transferred to a desiccator to cool and to prevent reabsorption of moisture from the surrounding air. The final weight of each bag was recorded, and the dry matter digestibility was calculated based on the difference between the initial sample weight and the residual weight after incubation, using the following formula: DMD (%) = (Sample weight (g) – Residue weight (g))/ (Sample weight (g))×100%.

Statistical analysis

The collected data, including pH value, ammonia nitrogen (N-NH_3) concentration, total volatile fatty acids (VFA), and dry matter digestibility (DMD), were analyzed using analysis of variance (ANOVA) according to a completely randomized design (CRD) consisting of four treatments and four replications. The significant effects of the treatment were further determined using Tukey's HSD (Honest Significant Difference) test was used for post hoc comparisons.

RESULTS

pH value of artificial rumen fluid inoculant

The culture process was carried out four times, each culture was incubated for 48 hours before the sub-culture was transferred to the next culture medium. Measurement of rumen fluid pH was carried out at the end of the incubation period. Based on the results of analysis of variance (ANOVA), it was found that the culture treatment had a significant effect ($P < 0.05$) on the pH value of the artificial rumen fluid. The average pH across cultures ranged from 6.67 ± 0.00 to 6.78 ± 0.02. Culture 4 recorded the highest pH (6.78 ± 0.02), which was significantly different from the other treatments. Meanwhile, Culture 3 (6.70 ± 0.03) was not significantly different from Culture 1 or Culture 2. However, Culture 1 (6.67 ± 0.00) was significantly different from Culture 2 (6.72 ± 0.04), indicating a gradual yet significant increase in pH with each successive culture.

N-NH_3 concentration of artificial rumen fluid inoculant

The concentration of N-NH_3 in artificial rumen fluid measured after 48 hours of incubation showed that the culture process had a significant effect ($P<0.05$) on the concentration of N-NH_3 in artificial rumen fluid. This can be seen from the graph in Figure 2. The highest average N-NH_3 concentration was observed in Culture 1, with a value of 21.88 ± 0.42 mM, which was significantly different ($P < 0.05$) from the other culture treatments. Culture 2 and Culture 3 recorded N-NH_3 concentrations of 12.87 ± 0.73 mM and 12.82 ± 0.52 mM, respectively, showed no significant difference between them; however, both were significantly lower than Culture 1 and significantly higher than Culture 4. The lowest N-NH_3 value was recorded in Culture 4 at 10.97 ± 0.26 mM, which was significantly different from all other treatments. The progressive decrease in N-NH_3 concentration from Culture 1 through Culture 4 suggests that the sequential culture process influences the availability and utilization of ammonia nitrogen in the artificial rumen fluid.

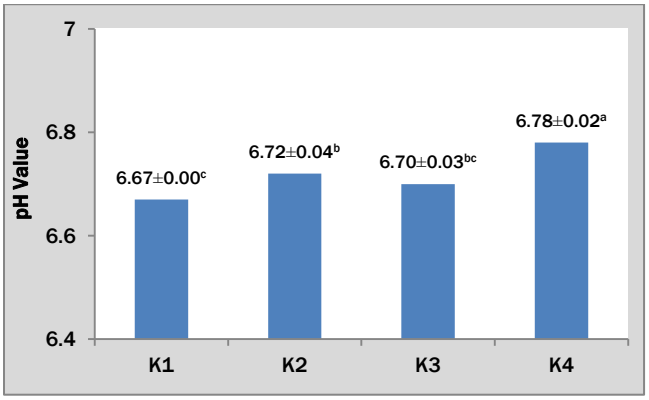

Figure 1 - Diagram of the effect of the culture process on the pH value of artificial rumen fluid. Different superscripts a, b and c on the pH value statistically indicate significant differences ($P < 0.05$). K1= Culture 1 (inoculant derived from rumen fluid), K2= Culture 2 (inoculant derived from culture 1), K3= Culture 3 (inoculant derived from culture 2), K4= Culture 4 (inoculant derived from culture 3).

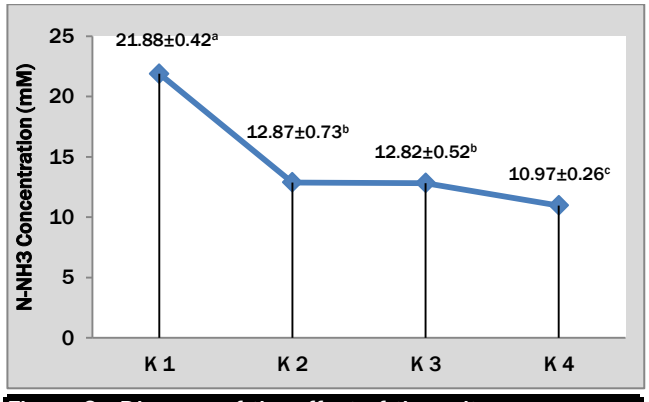

Figure 2 - Diagram of the effect of the culture process on the N-NH_3 concentration of artificial rumen fluid. Different superscripts a, b and c on N-NH_3 values statistically indicate significant differences ($P < 0.05$). K1= Culture 1 (inoculant derived from rumen fluid), K2= Culture 2 (inoculant derived from culture 1), K3= Culture 3 (inoculant derived from culture 2), K4= Culture 4 (inoculant derived from culture 3).

Total volatile fatty acid (VFA) production

Based on the results of analysis of variance, the production of total volatile fatty acids (VFA) in artificial rumen fluid cultured four times showed significant differences (P<0.05), as shown in Figure 3. The post hoc test results showed that the average VFA production in Culture 1 and Culture 2 was not significantly different, with values of 95.23 ± 5.02 mM and 95.23 ± 3.24 mM, respectively. However, both cultures produced significantly lower VFA concentrations than Culture 3 and Culture 4. VFA production began to increase in Culture 3 (106.24 ± 3.87 mM) and peaked in Culture 4 (111.36 ± 5.44 mM), although the difference between these two cultures was not statistically significant. Overall, these results indicate that the sequential culture process significantly influenced total VFA production, with a notable increase occurring after the second culture stage.

Dry matter digestibility of feed samples

The degradation of dry matter is very influential on the fulfillment of the energy source of microorganisms in the manufacture of artificial rumen fluid. The results of the dry matter degradation analysis can be seen in Figure 4 which presents a graph of dry matter degradation. The graph illustrates that dry matter degradation increased progressively with each stage of the culture process. Culture 1 recorded the lowest degradation value at 55.83 ± 0.92%, which was significantly different (P < 0.05) from Culture 3 and Culture 4, which showed the highest degradation value at 68,47 ± 3,39% and 70.69 ± 9.22%. Culture 2 showed an increase to 61.27 ± 5.49%, though this was not significantly different from all cultures. Culture 3 further increased to 68.47 ± 3.39%, showing a significant difference from Culture 1 but not from Cultures 2 or 4. Overall, the trend indicates that the sequential culture process positively influenced dry matter degradation, with significant improvements observed particularly in Culture 3 and Culture 4.

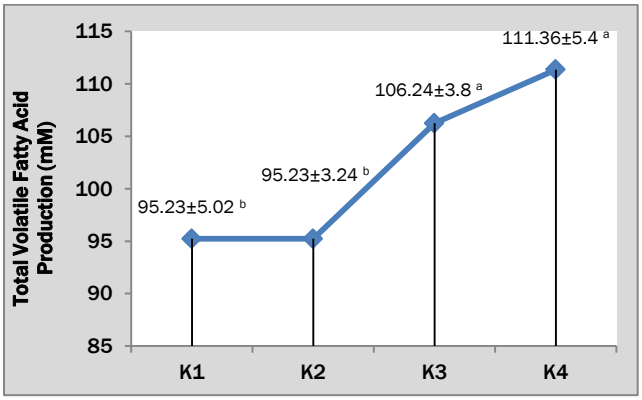

Figure 3 - Diagram of the effect of culture process on the production of total volatile fatty acids (VFA) in artificial rumen fluid. Different superscripts a and b on VFA values statistically showed significant differences (P<0.05). K1= Culture 1 (inoculant derived from rumen fluid), K2= Culture 2 (inoculant derived from culture 1), K3= Culture 3 (inoculant derived from culture 2), K4= Culture 4 (inoculant derived from culture 3).

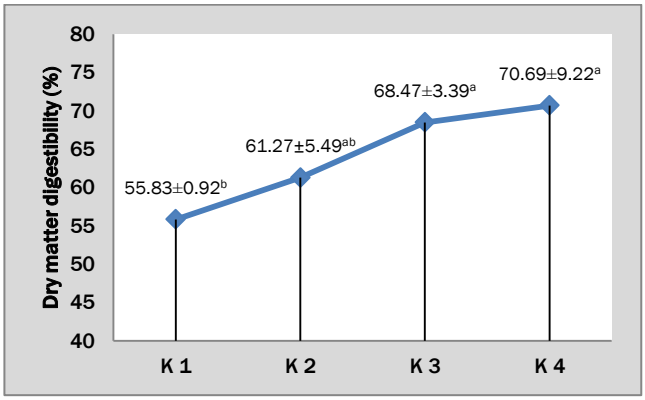

Figure 4 - Diagram of the effect of the culture process on the digestibility of feed dry matter in artificial rumen fluid. Different superscripts a and b on the degradation value of feed dry matter statistically showed significant differences (P < 0.05). K1= Culture 1 (inoculant derived from rumen fluid), K2= Culture 2 (inoculant derived from culture 1), K3= Culture 3 (inoculant derived from culture 2), K4= Culture 4 (inoculant derived from culture 3).

DISCUSSION

pH value of artificial rumen fluid inoculant

pH is a critical parameter in rumen fermentation, as it influences microbial growth, survival, and metabolic activity. According to Lund et al. (2020), pH affects the environmental conditions required for microbial proliferation. Jin and Kirk (2018) further explained that pH can alter microbial metabolic pathways by influencing cell surface interactions and enzyme activity. In this study, the pH values of artificial rumen fluid ranged from 6.67 in Culture 1 to 6.78 in Culture 4. These values fall within the optimal range of 5.5–7.0 for rumen fermentation, as reported by Öztürk and Gur (2021), indicating a suitable environment for microbial degradation of feed throughout the sequential cultures.

An upward trend in pH was observed across the culture stages, with a significant increase noted in the final culture. This suggests that the sequential culturing process influenced acid-base dynamics within the fermentation system, possibly due to shifts in microbial composition and fermentation by-products. Shen et al. (2023) noted that rumen pH is modulated by factors such as forage-to-concentrate ratio and the presence of buffering agents like bicarbonate, calcium carbonate, and magnesium oxide. In this study, artificial saliva based on McDougall's solution was added at each stage to maintain pH stability. This buffer rich in sodium bicarbonate helps sustain near-neutral pH conditions favorable for microbial activity (McDougall, 1948). Camacho et al. (2019) emphasized that the buffering capacity of McDougall's solution depends on both sodium bicarbonate content and CO_2 infusion to displace oxygen and maintain anaerobic conditions. The consistent application of this buffer and CO_2 flushing in every stage likely contributed to the observed pH

stability and gradual increase.

N-NH_3 concentration of artificial rumen fluid

Ammonia nitrogen (N-NH_3) concentration is a key indicator of nitrogen metabolism and microbial protein synthesis in the rumen. The metabolic activity of rumen microbiota plays a central role in nitrogen recycling, particularly through the utilization of ammonia as a primary nitrogen source (Hartinger et al., 2018). Approximately 80% of rumen bacteria rely on ammonia for their nitrogen requirements (Zurak et al., 2023). In this study, the highest N-NH_3 concentration was observed in Culture 1 (21.88 ± 0.42 mM), which can be attributed to the initial microbial adaptation phase. During this stage, residual nitrogenous compounds from the original rumen fluid—such as amino acids and soluble proteins—may have contributed to the elevated ammonia levels. This finding aligns with Zurak et al. (2023), who noted that ammonia in the rumen is produced from the microbial degradation of dietary proteins and amino acids.

As the culture progressed from Culture 2 to Culture 4, a gradual decrease in N-NH_3 concentration was observed. This trend suggests that microbes became more efficient in utilizing ammonia for microbial protein synthesis. Sari et al. (2021) stated that decreasing ammonia concentrations in fermentation media are indicative of increased microbial uptake for anabolic processes. Similarly, Silviani et al. (2024) emphasized that microbial protein synthesis is directly influenced by the availability of ammonia and the consumption of digestible dry matter, which supplies the energy needed for microbial growth.

The observed N-NH_3 concentrations, ranging from 21.88 mM in Culture 1 to 10.97 mM in Culture 4, remained within the optimal range of 6 to 21 mM reported by Suryani et al. (2020) for supporting rumen microbial activity. This indicates that despite the decreasing trend, the artificial rumen environment remained suitable for sustaining microbial metabolism throughout the sequential cultures.

Total volatile fatty acid (VFA) production

Total volatile fatty acids (VFAs), also known as short-chain fatty acids, are the primary end-products of anaerobic microbial fermentation in the rumen (Hasan et al., 2015). These compounds play an essential role in maintaining optimal conditions for microbial growth and contribute significantly to the host animal's energy supply (Jian et al., 2016). In the context of *in vitro* rumen fermentation, the culture process aims to sustain microbial activity to ensure consistent VFA production. The results of this study showed that VFA production in the early stages of culturing (Culture 1 and Culture 2) did not differ significantly. This may be attributed to the microbial community still undergoing adaptation to the *in vitro* rumen environment. During this period, the microbes require time to re-establish their metabolic activity. Hu and Yu (2005) noted that feed must first be hydrolyzed into soluble carbohydrates before fermentation into VFAs can occur, highlighting the lag between inoculation and active fermentation.

As the culture progressed to later stages (Culture 3 and Culture 4), a significant increase in VFA concentration was observed. This suggests that once adapted, microbial populations become more efficient at fermenting substrates. Alabi et al. (2023) reported that anaerobic microbes degrade plant lignocellulosic materials through fermentation, resulting in the production of VFAs. In this study, the increasing trend in VFA concentration was likely also influenced by the closed nature of the *in vitro* system, where VFAs are not absorbed as they would be *in vivo* through the rumen wall. Nozière et al. (2011) stated that in ruminants, VFAs are typically absorbed across the rumen epithelium and utilized as a major energy source. The average VFA concentrations observed in this study ranged from 95.23 mM in Culture 1 and 2, to 111.36 mM in Culture 4. These values fall within the optimal range for efficient microbial fermentation, typically between 70 and 150 mM (McDonald, 2010). Tunkala et al. (2022) similarly reported that fresh rumen fluid produced VFA concentrations ranging from 84.6 to 113.7 mM, which further supports the validity of the values obtained in this study. The stable and adequate VFA production across all cultures may also be supported by the presence of protein-rich feed components that are resistant to rapid degradation and the continuous use of buffering agents to stabilize fermentation conditions.

Dry matter digestibility of feed samples

Dry matter digestibility is a key indicator of microbial activity in the rumen and reflects the efficiency of feed degradation in the fermentation system (Moon et al., 2010). In the early stages of culture, rumen microorganisms still adapt to the artificial environment and feed substrate, which may result in suboptimal digestibility. This is consistent with findings by McDermott et al. (2020), who observed that in the first stage of the consecutive batch culture (CBC) method, dry matter digestibility was lower than subsequent cultures as microbial populations gradually adapted and increased their enzymatic activity. Rumen feed digestibility is largely determined by the ability of microbial enzymes to hydrolyze feed components, particularly structural carbohydrates (Castillo and Hernández, 2021). This study observed a progressive increase in dry matter digestibility across the sequential culture stages, indicating improved microbial adaptation and fermentative efficiency. Badarina et al. (2023) noted that feed can be categorized as having good digestibility when it reaches at least 60%. In this context, digestibility values observed after the initial culture stage in this study were in line with or exceeded that threshold, suggesting effective microbial utilization of feed substrates in the later cultures. Dry

matter digestibility reflects microbial activity and plays a critical role in supporting microbial growth and, ultimately, the nutrient availability for the host animal. The trend observed in this study is further supported by the concurrent increase in volatile fatty acid (VFA) production as culture stages progressed.

Dry matter comprises various organic constituents, primarily carbohydrates such as cellulose and hemicellulose (Palangi and Macit, 2019). As rumen microorganisms degrade these complex lignocellulosic structures into simpler polysaccharides, they generate VFAs as primary fermentation end-products (Palmonari et al., 2024). Thus, the positive correlation between increasing dry matter digestibility and VFA production observed in this study suggests that more substrate became available for microbial fermentation in the later culture stages, enhancing energy yield and microbial activity.

CONCLUSION

Sequential culturing of rumen fluid up to the fourth stage successfully maintained microbial viability and fermentative capacity for *in vitro* feed evaluation. The consistent increase in dry matter digestibility, volatile fatty acid production, stable pH, and optimal ammonia-N concentrations indicate that the microbial ecosystem remains functionally robust across culture cycles. These findings demonstrate that sequentially cultured rumen fluid can be a viable and sustainable inoculant alternative to fresh rumen fluid. The approach reduces dependence on fistulated animals, minimizes ethical concerns, and enhances reproducibility in laboratory-scale fermentation trials.

DECLARATIONS

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Data availability

The data generated and/or analyzed in this study can be obtained from the corresponding author upon reasonable request.

Authors' contribution

All authors contributed equally to the conception, design, data collection, analysis, and writing of the manuscript. All authors read and approved the final manuscript.

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Consent to publish

All authors agree to the publication of this manuscript.

Competing interests

The authors have not declared any conflict of interest.

REFERENCES

Alabi JO, Okedoyin DO, Anotaenwere CC, Wuaku M, Gray D, Adelusi OO, et al. (2023). Essential oil blends with or without fumaric acid influenced *in vitro* rumen fermentation, greenhouse gas emission, and volatile fatty acids production of a total mixed ration. Ruminants, 3(4):373-384. https://doi.org/10.3390/ruminants3040031

Badarina I, Dwatmadji D, and Rapi R (2023). *In vitro* digestibility and rumen ph of diet comprised by different level of *Indigofera zollingeriana* and *Pennisetum purpureum*. E3S Web of Conferences, 373: 01009. https://doi.org/10.1051/e3sconf/202337301009

Camacho LF, Silva TE, Palma MNN, Assunção AS, Rodrigues JP, Silva LFC, et al. (2019). Evaluation of buffer solutions and urea addition for estimating the *in vitro* digestibility of feeds. Journal of Animal Science, 97(2): 922–931. https://doi.org/10.1093/jas/sky464

Carro MD, Lebzien P, and Rohr K (1995). Effects of pore size of nylon bags and dilution rate on fermentation parameters in a semi-continuous artificial rumen. Small Ruminant Research 15(2): 113–119. https://doi.org/10.1016/0921-4488(94)00015-Y

Castillo C, and Hernández J (2021). Ruminal fistulation and cannulation: a necessary procedure for the advancement of biotechnological research in ruminants. Animals 11(7): 1-13. https://doi.org/10.3390/ani11071870

Castillo-González AR, Burrola-Barraza ME, Domínguez-Viveros J, and Chávez-Martínez A (2014). Rumen microorganisms and fermentation microorganismos y fermentacion ruminal. Archivos de Medicina Veterinaria, 46: 349–361. https://doi.org/10.4067/S0301-732X2014000300003

Close WH, and Karl-Heinz M (1986). Selected Topics in Animal Nutrition: A Manual Prepared for the 3rd Hohenheim Course on Animal Nutrition in the Tropics and Semi-Tropics. Germany: Deutsche Stiftung für internationale Entwicklung, Zentralstelle für Ernährung und Landwirtschaft. Selected topics in animal nutrition: a manual prepared for the 3rd Hohenheim ... - William H. Close, Karl-Heinz Menke - Google Buku

Covington AK, Bates RG, Durst RA (1985). Definition of ph scales, standard reference values, measurement of ph and related terminology (recommendations 1984). Pure and Applied Chemistry. 57: 531–542. https://doi.org/10.1351/pac198557030531

Fortina R, Patrucco SG, Barbera S, and Tassone S (2022). Rumen fluid from slaughtered animals: a standardized procedure for sampling, storage and use in digestibility trials. Methods and Protocols 5(4):1-10. https://doi.org/10.3390/mps5040059

Gascoyne DJ, and Theodorou MK (1988). Consecutive batch culture - a novel technique for the *in vitro* study of mixed microbial populations from the rumen. Animal Feed Science and Technology 21(2–4): 183–189. https://doi.org/10.1016/0377-8401(88)90099-5

Getachew G, Crovetto GM, Fondevila M, Krishnamoorthy U, Singh B, Spanghero M, et al. (2002). Laboratory variation of 24 h *in vitro* gas production and estimated metabolizable energy values of ruminant feeds. Animal Feed Science and Technology, 102(1–4): 169–180. https://doi.org/10.1016/S0377-8401(02)00212-2

Hartinger T, Gresner N, and Südekum K (2018). Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. Journal of Animal Science and Biotechnology, 9(1): 1–21. https://doi.org/10.1186/s40104-018-0249-x

Hasan SDM, Giongo C, Fiorese ML, Gomes SD, Ferrari TC, and Savoldi TE (2015). Volatile fatty acids production from anaerobic treatment of cassava waste water: effect of temperature and alkalinity. Environmental Technology (United Kingdom), 36(20): 2637–2646. https://doi.org/10.1080/09593330.2015.1041426

Hu Z and Yu H (2005). Application of rumen microorganisms for enhanced anaerobic fermentation of corn stover. Process Biochemistry, 40(7): 2371–2377. https://doi.org/10.1016/j.procbio.2004.09.021

Ji S, Zhang H, Yan H, Azarfar A, Shi H, Alugongo G, et al. (2017). Comparison of rumen bacteria distribution in original rumen digesta, rumen liquid and solid fractions in lactating holstein cows. Journal of Animal Science and Biotechnology, 8(1): 1–7. https://doi.org/10.1186/s40104-017-0142-z

Jian G, Meng-zhi W, Yu-jia J, Xue-zhao S, Tian-yi W, and Liang-feng S (2016). Impacts of the unsaturation degree of long-chain fatty acids on the volatile fatty acid profiles of rumen microbial fermentation in goats *in vitro*. Journal of Integrative Agriculture 15(12): 2827-2833. https://doi.org/10.1016/S2095-3119(16)61418-1

Jin Q, and Kirk MF (2018). Ph as a primary control in environmental microbiology: 2. Kinetic perspective. Frontiers in Environmental Science, 6(9): 1–16. https://doi.org/10.3389/fenvs.2018.00101

Kera F, Urge M, Animut G, and Tolera A (2022). Effects of inclusion of different levels of ethiopian thyme (*Thymus schimperi* ronniger) as natural additive on chemical composition and *in sacco* dry matter degradability of total mixed ration and feed ingredients. Open Journal of Veterinary Medicine, 12(11): 155–169. https://doi.org/10.4236/ojvm.2022.1211013

Kromann RP, Meyer JH, and Stielau WJ (1967). Steam distillation of volatile fatty acids in rumen ingesta. Journal of Dairy Science, 50(1): 73–76. https://doi.org/10.3168/jds.S0022-0302(67)87356-9

Lodge-Ivey SL, Browne-Silva J, and Horvath MB (2009). Technical note: bacterial diversity and fermentation end products in rumen fluid samples collected via oral lavage or rumen cannula. Journal of Animal Science, 87(7): 2333–2337. https://doi.org/10.2527/jas.2008-1472

Lund PA, Biase DD, Liran O, Scheler O, Mira NP, Cetecioglu Z, Fernández EN, et al. (2020). Understanding how microorganisms respond to acid ph is central to their control and successful exploitation. Frontiers in Microbiology ,11(9): 1-8. https://doi.org/10.3389/fmicb.2020.556140

Mbiriri DT, Cho S., Mamvura CI, and Choi N (2016). Assessment of rumen microbial adaptation to garlic oil, carvacrol and thymol using the consecutive batch culture system. Journal of Veterinary Science and Animal Husbandry 4(1): 1–7. https://doi.org/10.15744/2348-9790.4.101

McDermott K, Lee MRF, McDowall KJ and Greathead HMR (2020). Cross inoculation of rumen fluid to improve dry matter disappearance and its effect on bacterial composition using an *in vitro* batch culture model. Frontiers in Microbiology, 11(9): 1-18. https://doi.org/10.3389/fmicb.2020.531404

McDonald P (2010). Animal Nutrition. Seventh ed. England: Pearson Education Limited. https://api.pageplace.de/preview/DT0400.9781408204276_A25051376/preview-9781408204276_A25051376.pdf

McDougall EI (1948). Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochemical Journal, 43(1): 99–109. https://doi.org/10.1042/bj0430099

Moon YH, Ok UJ, Lee SJ, Ha JK, and Lee SS (2010). A comparative study on the rumen microbial populations, hydrolytic enzyme activities and dry matter degradability between different species of ruminant. Animal Science Journal, 81(6): 642–647. https://doi.org/10.1111/j.1740-0929.2010.00782.x

Nozière P, Glasser F, and Sauvant D (2011). *In vivo* production and molar percentages of volatile fatty acids in the rumen: a quantitative review by an empirical approach. Animal, 5(3): 403–414. https://doi.org/10.1017/S1751731110002016

Öztürk H, and Gur G (2021). Rumen physiology: microorganisms, fermentation and manipulation. Ankara Universitesi Veteriner Fakultesi Dergisi, 68(4): 423–434. https://doi.org/10.33988/auvfd.960447

Palangi V, and Macit M (2019). *In situ* crude protein and dry matter ruminal degradability of heat-treated barley. Revue de Medecine Veterinaire, 170(7): 123–128. https://envt.hal.science/hal-04931351v1/document

Palma-Hidalgo JM, Jiménez E, Popova M, Morgavi DP, Martín-García AI, Yáñez-Ruiz DR, et al. (2021). Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats. Animal Microbiome, 3(1): 1-21. https://doi.org/10.1186/s42523-021-00073-9

Palmonari A, Federiconi A, and Formigoni A (2024). Animal board invited review: the effect of diet on rumen microbial composition in dairy cows. Animal, 18: 101319. https://doi.org/10.1016/j.animal.2024.101319

Raffrenato E, Badenhorst MJ, Shipandeni MNT, and Van ZWH (2021). Rumen fluid handling affects measurements of its enzymatic activity and *in vitro* digestibility. Animal Feed Science and Technology 280(6): 1–7. https://doi.org/10.1016/j.anifeedsci.2021.115060

Sari RWW, Jamarun N, Elihasridas, Yanti G (2021). In-vitro rument liquid characteristics (ph, vfa, and nh_3) from sugar cane top fermented with different levels of phanerochaete chrysophobia. Proceedings of the International Seminar on Promoting Local Resources for Sustainable Agriculture and Development (ISPLRSAD 2020), 13: 190–193. https://doi.org/10.2991/absr.k.210609.031

Shen J, Zheng W, Xu Y, and Yu Z (2023). The inhibition of high ammonia to *in vitro* rumen fermentation is ph dependent. Frontiers in Veterinary Science, 10(4):1-12. https://doi.org/10.3389/fvets.2023.1163021

Silva ÉBR , Silva JAR, Silva WC, Belo TS, Sousa CEL, Santos MRP, Neves KAL, et al. (2024). A review of the rumen microbiota and the different molecular techniques used to identify microorganisms found in the rumen fluid of ruminants. Animals 14(10): 1-20. https://doi.org/10.3390/ani14101448

Silviani E, Yasmin N, Martin RSH, and Jayanegara A (2024). Urea-nitrate coating with zeolite reduces *in vitro* ammonia concentration in the rumen. Iop Conference Series: Earth and Environmental Science 1359(1): 1-5. https://doi.org/10.1088/1755-1315/1359/1/012110

Spanghero M, Chiaravalli M, Colombini S, Fabro C, Froldi F, Mason F, Moschini M, et al. (2019). Rumen inoculum collected from cows at slaughter or from a continuous fermenter and preserved in warm, refrigerated, chilled or freeze-dried environments for *in vitro* tests. Animals, 9(10): 1–14. https://doi.org/10.3390/ani9100815

Suryani NN, Suama IW, Mahardika IG, and Sarini NP (2020). Rumen fermentation and microbial protein synthesis of bali cattle heifers (*Bos sondaicus*) fed ration containing different energy protein level. Jurnal Sain Peternakan Indonesia, 15(2): 187–194. https://doi.org/10.31186/jspi.id.15.2.187-194

Tassone S, Fortina R, and Peiretti PG (2020). *In vitro* techniques using the daisyii incubator for the assessment of digestibility: a review. Animals, 10(5): 1–24. https://doi.org/10.3390/ani10050775

Thirumalaisamy G, Malik PK, Trivedi S, Kolte AP, and Bhatta R (2022). Effect of long-term supplementation with silkworm pupae oil on the methane yield, ruminal protozoa, and archaea community in sheep. Frontiers in Microbiology, 13(3): 1–14. https://doi.org/10.3389/fmicb.2022.780073

Tilley JMA, and Terry RA (1963). A two-stage technique for the *in vitro* digestion of forage crops. Grass and Forage Science, 18(2): 104–11. https://doi.org/10.1111/j.1365-2494.1963.tb00335.x

Tunkala BZ, DiGiacomo K, Hess PSA, Dunshea FR, and Leury BJ (2022). Rumen fluid preservation for *in vitro* gas production systems. Animal Feed Science and Technology, 292(8):1-9. https://doi.org/10.1016/j.anifeedsci.2022.115405

Vinyard JR, and Faciola AP (2022). Unraveling the pros and cons of various *in vitro* methodologies for ruminant nutrition: a review. Translational Animal Science 6(4): 1–9. https://doi.org/10.1093/tas/txac130

Yáñez-Ruiz DR, Bannink A, Dijkstra J, Kebreab E, Morgavi DP, O'Kiely P, et al. (2016). Design, implementation and interpretation of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants-a review. Animal Feed Science and Technology 216: 1–18. https://doi.org/10.1016/j.anifeedsci.2016.03.016

Zewdie AK (2019). Ec nutrition review article the different methods of measuring feed digestibility: a review. Ec Nutrition, 14(1): 68–74. https://www.researchgate.net/publication/330103516

Zurak D, Kljak K, and Aladrović J (2023). Metabolism and utilisation of non-protein nitrogen compounds in ruminants : a review. Journal of Central European Agriculture, 24(1): 1–14. https://doi.org/10.5513/JCEA01/24.1.3645