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The Effect of Saliva with Different Nitrogen Compositions on Ruminal Fermentation in a Rumen Simulator Technique (Rusitec®) System Fed a Lactating Dairy Cow Diet

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Abstract: In vitro methods have advanced research on rumen microbiology and fermentation. However, artificial saliva formulation may need adjustments, particularly in urea content, for modern diets, warranting further research. This study investigated the effects of different nitrogen (N) levels in artificial saliva on ruminal fermentation and digestion in diets for dairy cows using a Rusitec® system. Eighteen fermenters tested three saliva treatments with different N levels: a standard saliva as the control and two treatments with N reduced by 15% and 30%. Data were analyzed as a completely randomized design using the MIXED procedure of SAS (v. 9.4), with linear and quadratic contrasts tested for treatment effects (significance set at $p \leq 0.05$). Results showed that altering N content had no significant effect on pH, ammonia concentrations, or NH_3-N outflow, nutrient digestibility (dry matter, crude protein, fiber, and starch), gas and methane production, or volatile fatty acid concentrations. The efficiency of microbial protein synthesis and N flow exhibited quadratic responses, with the lowest values observed at the highest level of N reduction in the saliva (−30%). These findings suggest that although ruminal function and digestion remain stable with reduced N, microbial protein synthesis efficiency may decline beyond a threshold.

Keywords: diet manipulation; efficiency; in vitro; methane emissions; ruminants

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Academic Editor: Qing Zhang

Received: 28 April 2025
Revised: 22 May 2025
Accepted: 5 June 2025
Published: 11 June 2025

Citation: Castro, Í.R.R.d.; Silva, L.d.N.C.d.; Carrari, I.F.; Leite, G.B.C.; Paula, E.M.d.; Cezar, A.M.; Marcondes, M.I. The Effect of Saliva with Different Nitrogen Compositions on Ruminal Fermentation in a Rumen Simulator Technique (Rusitec®) System Fed a Lactating Dairy Cow Diet. *Fermentation* **2025**, *11*, 340. https://doi.org/10.3390/fermentation11060340

1. Introduction

Applying methodologies that provide nutritional insights into animal feeds and their metabolic interactions allows researchers to predict how specific dietary components can enhance productivity and minimize losses from metabolic disorders [1,2]. As a result, this optimization maximizes the efficiency of utilizing nutritional resources in animal production systems [3]. In vivo studies, however, require a substantial number of homogeneous

animals maintained throughout adaptation and sampling, making them expensive, labor-intensive, and time-consuming [4]. The inherent complexity of the rumen also challenges controlled experimentation under in vivo conditions.

In vitro methodologies in animal nutrition have proven valuable due to their high precision (or low variance) compared with in vivo systems, lower cost, quicker results acquisition, efficient environmental control, ability to work with a wide range of treatments, and smaller sample sizes [5]. As a result, these alternative techniques have significantly enhanced research capacity and expanded the understanding of rumen microbiology and the biological processes involved in ruminal fermentation [4,6].

In ruminant research, for an in vitro system to be reliable, artificial rumen must fulfill certain criteria to be considered an appropriate experimental tool. It should emulate the natural rumen in various aspects, including the physical environment (such as temperature, pH, turnover rates, etc.) and the maintenance of essential microbial populations [7,8]. Additionally, artificial rumen should replicate nitrogen (N) recycling, as this process changes with diet, particularly with dietary N content [9].

To create an environment within the Rumen Simulator Technique (Rusitec®) system that resembles the rumen, McDougall [10] developed a composition of artificial saliva that many trials have adopted. Recent research, however, suggests that McDougall's artificial saliva may require modification when applied to modern diets [11,12]. These diets, with varying N content, influence ammonia and nitrogen flows, both of which are critical for microbial N efficiency and energy use in the rumen. An imbalance in N supply can disturb these dynamics, compromising microbial protein synthesis and fermentation efficiency [11,12].

Brandao and Faciola [13] revealed that when feeding the fermenters with diets rich in crude protein (CP), such as dairy cow diets, the continuous addition of urea via saliva could lead to higher in vitro ammonia–nitrogen (NH_3-N) accumulation than in vivo omasum sampling studies with similar diets. When examining microbial efficiency and nitrogen (N) metabolism, Brooks et al. [14] found that excessive N in the rumen environment leads to increased energy expenditure by ruminal microbes. This N buildup may disrupt fermentation processes, reducing the total volatile fatty acid (VFA) concentration and producing inaccurate N metabolism data. Moreover, in high-N diets, the inclusion of urea could introduce more NH_3-N than microbes can efficiently convert into microbial N, affecting overall fermentation dynamics [15]. Consequently, maintaining a fixed N content in artificial saliva across different diets may require adjustments. Addressing this concern would enhance the reliability of artificial rumen systems as valuable tools for studying ruminant nutrition and microbial fermentation processes.

Given the potential for excessive N in artificial saliva to disrupt fermentation dynamics and microbial efficiency, optimizing urea concentration in in vitro systems is critical. Therefore, we hypothesized that a reduced level of N inclusion in the artificial saliva would not affect the fermentation and microbial protein production in in vitro continuous flow systems. This study aimed to evaluate three levels of N inclusion in the artificial saliva on pH, ammonia nitrogen concentration and outflow, VFA profiles, gas and methane production, nutrient digestibility [dry matter (DM), organic matter (OM), CP, neutral detergent fiber (NDF), and starch], and microbial protein synthesis efficiency using a continuous flow system (Rusitec®).

2. Materials and Methods

The experiment was conducted at Washington State University in Pullman, Washington, DC, USA. The ruminal content donor animals utilized in this study were handled by

the guidelines set by Washington State's Institutional Animal Care and Use Committee (WSU/IACUC; ASAF #6608).

2.1. Experimental Design and Treatments

The experiment followed a completely randomized design with three treatments, representing different amounts of N in the saliva. The study used 18 Rusitec® fermenters, with six fermenters assigned to each treatment group. The methodology for the experiment was adopted from the work of Czerkawski and Breckenridge [16]. The experiment lasted 10 days, during which the fermenters were allowed to reach a steady state over the initial 7 days. Subsequently, a sampling period of 3 days (day 8 to 10) was conducted.

The treatments involved using saliva with varying N amounts, expressed as proportions of the artificial saliva composition based on McDougall's [10] formulation. The three treatment groups were as follows:

(1) Control: 100% of the artificial saliva composition by McDougall [10] (0.300 g of N/L).
(2) −15% N: Saliva with a 15% decrease in N content (0.255 g of N/L).
(3) −30% N: Saliva with a 30% decrease in N content (0.210 g of N/L).

Throughout the experiment, the Rusitec fermenters were fed a total mixed ration (TMR) diet, the composition of which can be found in Table 1. The TMR used in the experiment was originally formulated to contain 50% DM, typical of diets fed to lactating dairy cows in vivo. However, to ensure uniformity, wet feeds such as silages were dried at 55 °C and then ground through a 4 mm screen using a Willey mill (model 4, Philadelphia, PA, USA), and the diet was then placed in polyester bags for incubation. This pre-processing step increases the overall DM content of the diet, diverging from its original in vivo formulation.

Table 1. Ingredient chemical composition (% of DM unless otherwise noted in the total mixed ratio diet containing triticale silage).

Ingredient Composition	% DM
Corn silage	24.20
Alfalfa hay	12.52
Triticale silage	12.27
Corn ground fine	18.78
Soybean meal, 48%	7.19
Urea	1.06
Barley	8.35
Rice hulls	14.49
Mineral mixture [1]	1.15
Chemical composition	
Dry matter (DM, % of as fed)	91.87
Crude protein (CP)	13.37
Non-fibrous carbohydrate (NFC) [2]	39.57
Neutral detergent fiber (NDF)	36.14
Ether-extract (EE)	2.90 *
Ash	8.02
Starch	37.02
Metabolizable energy (ME),[3] Mcal/kg of DM	2.43
CP–ME ratio (g/Mcal)	5.50

[1] Contained per kilogram of the supplement: 0.0079 of vitamin A 30S, 0.0021 of vitamin D 30S, 1.1263 g of vitamin E Lutavin 50, 103.03 g of Ammonium sulfate, 209.54 g of Limestone, 264.91 g of Dicalcium phosphate, 421.30 g of Salt (iodized, 0.01%), 0.0159 g of Cobalt carbonate and 0.0562 g of Sodium selenite. * Estimated value. [2] NFC = 100 − (CP + NDF + EE + Ash) [17]. [3] Calculated from NRC [17].

2.2. Experimental Apparatuses and Incubations

The rumen simulation system used in this study was custom-designed and constructed at the Washington State University Department of Animal Science Farm Shop. This system was based on the original Rusitec® model developed by Czerkawski and Breckenridge [16], but it featured some modifications to suit the specific experimental requirements. In contrast to the original design, our adapted apparatus comprised 18 fermenters, organized into three lines, each containing six fermenters. Each fermenter had a capacity of 2200 mL and was equipped with an artificial saliva inflow positioned at the top of the fermenter and a gas outflow.

To maintain a controlled and consistent temperature environment, all fermenters were immersed in a water bath with a total volume of approximately 400 L. The water bath was equipped with two immersion-circulating heaters, which worked in tandem to ensure a stable temperature of 39 $\pm$ 0.5 °C throughout the experiment.

The three treatments were randomly assigned within the Rusitec apparatus (six replications per treatment). Fermenters were gas-tight with a cap that, when fixed, maintained the fermentation vessels thermodynamically as a closed system. Located on the top of caps, each fermenter had an inlet for artificial saliva, an outlet for the gas that accumulated in the headspace, and an outlet for liquid effluent.

To facilitate the collection and storage of gas samples over a 24 h period, gas outlets from the fermenters were linked to 5 L Tedlar propylene sampling bags (Environmental Samply Supply Inc., Oakland, CA, USA; [18]). The connection between the outlets and the sampling bags was made using Tygon tubing with dimensions of 1/16 inch ID $\times$ 1/8-inch OD (VWR Scientific®, model 1370 GM, Radnor, PA, USA).

The liquid effluent produced during the fermentation process was collected and stored in 2 L plastic containers (Nalgene®, Thermo Fisher Scientific Inc., Bohemia, NY, USA), connected to each fermenter using Tygon tubing. The Tygon tubing used for this purpose had 3/8-inch ID $\times$ 1/2-inch OD dimensions and was sourced from VWR Scientific® (model 1370 GM, Radnor, PA, USA). The containers were kept on a constant ice bath to stop microbial activity and were measured daily for volume and weight. This effluent collection allowed for monitoring the fermentation outputs and assessing the changes in the rumen ecosystem over time.

To supply the fermenters with artificial saliva, a hydraulic pump (Watson-Marlow®, model 205U, Cornwall, UK) was employed in the experimental setup to ensure a controlled and consistent flow of artificial saliva to the fermenters throughout the experiment. The pump was connected to the fermenters using Tygon tubing, which had 1/16-inch ID $\times$ 1/8-inch OD dimensions and was sourced from VWR Scientific® (model 1370 GM, Radnor, PA, USA). The saliva flow was checked daily to ensure the tubes were clear. Every 5 days, the tubes were cleaned with hot water to remove any clogs. Additionally, every other day, the 1.1 mL/min flow rate was verified to ensure it remained consistent.

The experiment was initiated by filling each fermenter with 2200 mL of rumen fluid, following the methodology outlined by Ribeiro et al. [19]. To obtain the cattle inoculum, rumen fluid was collected from the ventral, central, and dorsal areas of the rumen of two cannulated Angus cows with an average body weight of 759.77 $\pm$ 64.15 kg. These cows were housed at the Beef Cattle Center facility and were fed a mixed ration of chopped forages (55% alfalfa, 25% straw, and 10% hay barley) with 10% water added, resulting in a forage-to-concentrate ratio of 52:48.

Rumen fluid sampling took place approximately 3 h after the morning feeding. The collected ruminal content was carefully squeezed through four layers of cheesecloth and then transferred into eight preheated 6 L insulated containers, which were promptly trans-

ported to the laboratory. Furthermore, around 400 g of ruminal solid digesta (200 g coming from each donor cow) was collected to serve as the initial inoculum for the fermenters.

Upon arrival at the laboratory, the rumen contents obtained from the donor cows were pooled in equal volumes in a large pre-heated vessel and gently stirred. They went through an additional filtration step, passing through four layers of cheesecloth into an insulated thermos with constant N_2 inflow to maintain its anaerobic state. This pooled rumen fluid was utilized simultaneously for the inoculation of all fermenters. Finally, a 500 mL sample of the inoculum was collected and immediately frozen at −20 °C. This frozen sample served as a background reference for subsequent analyses conducted at the end of the experiment.

On day 1 of the experiment, rumen fluid inoculum was added into each fermenter, followed by a sample of solid digesta (20 g wet weight), placed in a prelabeled bag. A separate bag with diet (20 g wet weight) was also prepared. The vessels were infused with O_2-free N gas (N_2) to create an anaerobic environment representative of ruminal conditions, where O_2 is absent due to microbial activity. Once the anaerobic environment was established, the vessels were securely closed to maintain an O_2-free system throughout the experiment.

On day 1, the experiment was initiated, and the subsequent data collection and observation periods were conducted following the planned protocol. After a 24 h incubation period (day 2), the solid rumen digesta present in each fermenter was replaced with another bag containing the diet. From day 1 onwards, the fermenters now contained two bags with different incubation times. The feeding process involved daily openings of the fermenters at 1:00 P.M. to replace the bag that had been incubated for 48 h. This replacement of the bags ensured a continuous supply of fresh diet for the fermentation process, allowing for the study of microbial activities over successive incubation periods.

To maintain consistent conditions, artificial saliva with a pH of 8.2 was prepared daily. The artificial saliva was continuously infused into the fermenters at a dilution rate of 0.7 d^{-1} (1600 mL/d). The continuous infusion of artificial saliva ensured a steady supply of essential nutrients and fluids to support microbial fermentation throughout the experimental period [20].

Each fermentation unit was flushed with (N_2) during the bag exchange to help remove any traces of O_2, ensuring that the fermenters remained under strictly anaerobic conditions.

2.3. Measurements

2.3.1. In Vitro DM and Nutrients Digestibility

The true DM disappearance at 48 h was determined from days 8 to 10. Feed bags with the TMR diet were removed from each fermenter and dried at 55 °C for 72 h (VWR Scientific®, model 1370 GM, Radnor, PA, USA). Upon removal from the fermenters, bags with feed were gently squeezed to expel the excess liquid, and then washed until the water draining from the bags was clear. Then, the bags were placed in an air-forced oven set at 55 °C for 72 h to ensure complete drying and then for 2 h at 105 °C oven. The difference between the initial weight of the feed added to the bags and the final weight of the residues was used to calculate the true DM disappearance.

2.3.2. Fermentation Parameter

Gas bags were closed before opening the fermenters or effluent collection. Before the feed bag exchange, daily total gas production (d 1 to d 10) from each fermenter was measured using a flowmeter (Omega Engineering Inc., Stamford, CT, USA). The bag was connected to a vacuum pump (VacuMaster® model, Robinair, Warren, MI, USA) and the pressure gauge to determine gas volume by pressure difference. The vacuum pump was

started, and when pressure reading stabilized at 0.00 kPa, the gas valve was closed, and the value was registered. Gas production data from days 8 to 10 were averaged to be used in the statistical analysis. Also, during days 8 to 10, before taking total gas measurements, gas samples of 30 mL were collected from the septum of the collection bags using a 21-gauge needle. These samples were then carefully transferred into evacuated 30 mL syringes equipped with caps to preserve the integrity of the collected gas until methane (CH_4) analysis. Due to the elevated concentration of CH_4 in the samples, a serial dilution was necessary prior to analysis to bring methane levels within the detectable range of the GC. First, 1 mL of the CH_4-rich gas sample was mixed with 24 mL of oxygen-free nitrogen (N_2), resulting in a 25 mL mixture. Then, 1 mL of this mixture was further diluted with 120 mL of O_2-free N_2, yielding a final volume of 121 mL. This two-step serial dilution achieved a total dilution factor of 3025× (25 × 121), which ensured accurate quantification of CH_4 concentrations during analysis.

The pH of the fluid from each fermenter was recorded daily (d 1 to d 10) at the time of feed bag exchange using a pH meter (HI9813-5 model, Hanna Instruments, Smithfield, RI, USA). Measurements were taken using a graduated cylinder and a scale (Metter Toledo®, model PL1502E, Columbus, OH, USA) to measure the daily effluent production, volume, and weight.

For the analysis of VFA concentration in the fermenter (d 8 to 10), 8 mL subsamples were directly collected from the effluent flasks at the time of feed bag exchange. These subsamples were then stored in screw-cap vials and preserved with 2 mL of 25% (*w*/*w*) metaphosphoric acid, following the method by Giraldo et al. [21]. Simultaneously, 10 mL subsamples of effluent were collected and preserved with 100 µL of H_2SO_4 (50%, *vol*/*vol*) to determine NH_3-N, as also described in the study by Giraldo et al. [21].

Samples designated for the analysis of VFA and NH_3-N were centrifuged at 1000× *g* for 15 min at 4 °C, and the supernatants were carefully separated and isolated. After centrifugation, the samples were transferred to screw-cap vials and immediately frozen at −20 °C until the analysis.

The analysis of VFAs was performed using gas chromatography with flame ionization detection (GC-FID). Samples were centrifugated at 14,000 RPM for 5–10 min to separate the supernatant, which was then diluted with a 0.01 M phosphoric acid solution to achieve an appropriate concentration range. The diluted samples were filtered through a 0.45 µm syringe filter into 2 mL clear GC vials and capped with 9 mm PTFE/SIL blue caps. A multi-component VFA stock standard (4000 mg/L) was prepared using pure acids, including acetic, propionic, butyric, and others, diluted with 30% phosphoric acid. Calibration standards (100–4000 mg/L) were prepared by diluting the stock with 0.01 M phosphoric acid and stored frozen for up to four months. The GC-FID analysis followed a standardized operating procedure, ensuring proper instrument setup, method loading (VFA.M), and sequence table verification. Samples were injected using a dedicated VFA syringe, with blanks prepared using 0.01 M phosphoric acid. Data were processed using autointegration, and results were exported for further analysis. Ammonia nitrogen concentrations in the samples were quantified using a phenol-hypochlorite assay adapted from Berthelot [22] and Broderick and Kang [23]. Samples were centrifuged (10,000× *g*, 15 min, 4 °C) to remove particulates, and 20 µL aliquots were reacted sequentially with 1 mL phenol reagent (33 mL 90% phenol + 0.15 g sodium nitroprusside in 3 L H_2O) and 0.8 mL hypochlorite reagent (15 g NaOH + 150 mL 5.25% NaOCl + sodium phosphate buffer in 3 L H_2O). After 5 min incubation at 95 °C, 200 µL of the indophenol blue product was transferred to a 96-well plate and absorbance measured at 620 nm using a BioTek Synergy H1 Multimode Reader (Agilent®, Santa Clara, CA, USA). The concentrations of VFA and NH_3-N (mmol/L) were

then multiplied by the daily effluent production (L/d) to calculate the VFA and NH_3-N production rates (mmol/d).

2.3.3. Microbial Protein Synthesis

To label the bacteria in the fermenters, a ^{15}N isotope was used. On day 6 of the experiment, 0.024 g/L $(NH_4)_2SO_4$ in McDougall's buffer was replaced with an isonitrogenous amount of urea (0.024 g/L ^{15}N-enriched $(NH_4)_2SO_4$) in each treatment. This timing is applied to allow sufficient time for the fermenters to reach a isotopic steady-state ^{15}N enrichment of the NH_3 pool before the sampling period began on day 8. The ^{15}N-enriched $(NH_4)_2SO_4$ used in the experiment was obtained from Sigma Chemical Co., St. Louis, MO, USA, with a minimum ^{15}N enrichment of 10.01 atom %, following the method described by Calsamiglia et al. [24]. This replacement was carried out to achieve a steady-state ^{15}N enrichment of the NH_3 pool in the fermenters until the experiment's conclusion. Simultaneously, on the same day (day 6), samples of each saliva (both with and without ^{15}N) were collected to be used as background references. At the end of the feeding time on day 6, a 1 mL pulse dose of ^{15}N was infused into each fermenter, with a concentration of 0.024 g/L. This infusion was intended to label the NH_3-N pool within each fermenter, following the method outlined in the study by Brandao et al. [25].

Microbial isolation was carried out with a modified version of the methods developed by Krizsan et al. [26]. After the sampling period (d 10), the entire fermenter's liquid content was blended for 30 s. Subsequently, the remaining particles on the 4-layer cheesecloth were washed with 400 mL of 0.9% (*wt*/*vol*) NaCl solution, and the cheesecloth was squeezed to ensure maximum material recovery into the liquid-associated bacteria bottles.

The liquid samples were then subjected to a three-step centrifugation process using an RC-5B plus superspeed centrifuge (Sorvall®, Thermo Fisher Scientific Inc., Bohemia, NY, USA), as follows:

1. The first centrifugation was performed at 1000× *g* for 10 min at 5 °C to discard residual feed particles.
2. The supernatant from the first centrifugation was subjected to a second centrifugation step at 11,250× *g* for 20 mi at 5 °C. Afterward, the supernatant was carefully removed using plastic pipettes, and the resulting pellets were resuspended in 200 mL of McDougall's solution and thoroughly mixed using a lab spoon.
3. The material obtained from the second centrifugation was further processed in a third centrifugation step at 16,250× *g* for 20 mi at 5 °C. The final supernatant was discarded using a plastic pipette.

Lastly, the pellets were resuspended in distilled water, freeze-dried, and stored at −20 °C for subsequent analysis of ^{15}N enrichment, total N, and DM content.

2.3.4. Methane Analysis

Syringes containing the diluted gas samples were subjected to analysis using a Hewlett-Packard 5890 Series II Gas Chromatograph (Hewlett-Packard, Wilmington, DE, USA). The GC had a FID specifically designed for CH_4 analysis. Stainless steel columns with dimensions of 3.175 mm × 2.159 mm were utilized, and these columns were packed with Porapak N, with N_2 serving as the carrier gas.

The FID detector requires air and hydrogen (H) to ensure accurate and reliable results. Before initiating the gas analysis on the GC, the flame was allowed to burn for at least 30 min to achieve stabilization.

The gas flow rate during the analysis was 23 mL/min for Argon-CH_4. The column oven temperature was maintained at 50 °C, while the FID temperature was set to 250 °C

during analysis. These parameters were optimized to obtain precise measurements and consistent data for CH_4 analysis.

A Model 202 four-channel PeakSimple data system (SRI Instruments, Torrance, CA, USA) was used to acquire and transmit GC data to a computer. The corresponding PeakSimple software version 4.88 was read and automatically integrated the area under each peak after injection, reporting the results in area units (AU). Chromatographs were autozeroed during each sample injection, and both detectors had a run time of 60 min.

A series of standards, comprising a CH_4 standard (4.44 ppm), was analyzed. These standards were injected into the GC column using a 1.0 cubic capacity gas sample loop at a rate of 5 mL over a 5 s interval. Each standard was injected at least twice, and the accepted coefficient of variation was set at 3%. Once the sample chromatographs met this repeatability criterion, the canisters containing the unknown samples were analyzed. The unknown samples were injected at least twice over a 5 s interval.

Results were excluded from the analysis if the sample was mistakenly injected at an inconsistent rate, resulting in it being outside the expected range (defined as ± 3 standard deviations from the mean peak area of calibration standards). Additionally, peaks exhibiting doublet formation, shoulder peaks, or baseline distortions were rejected to ensure analytical reliability. Manual integration was applied only when automated software integration failed to correctly identify the baseline, as recommended in studies on GC-based methane quantification.

A comparison was made with the known standards to calculate the CH_4 concentration in the unknown samples, typically using 4.02 ppm for CH_4. The following ratio was utilized for this purpose:

$$(\text{concentration standard}/\text{AUstandard}) = (\text{concentration unknown}/\text{AU unknown}). \quad (1)$$

The concentrations were converted to mg/m^3 using the formula stated in Boguski [27]:

$$\text{Concentration } (mg/m^3) = 0.0409 \times \text{concentration (ppm)} \times \text{molecular weight} \quad (2)$$

where the value 0.0409 is the inverse of the gas constant R (0.0821 $L{\cdot}atm{\cdot}mole^{-1}{\cdot}^{\circ}K$) multiplied by the standard ambient temperature (298 °K).

The CH_4 concentration was divided by the corresponding sampling period's daily average wind speed (WS) to consider atmospheric dilution during canister sampling.

2.3.5. Chemical Analysis

Bags with diet residues were pre-dried at 55 °C for 72 h using a VWR Scientific® model 1370 GM oven (Radnor, PA, USA). These dried residues were then pooled over three days (d 8, 9, and 10 for each fermenter) to ensure an adequate sample amount for chemical analysis. Before analysis, the samples were ground through a 1 mm screen in a Wiley mill (standard model 4; Arthur H. Thomas Co., Philadelphia, PA, USA).

Substrates underwent chemical analysis for DM (method no. 930.15) and ash content (method no. 942.05) following AOAC International [28] protocols. The concentration of NDF was determined using a heat-stable amylase, with residual ash content included, as per the method described by Van Soest et al. [29]. The total N concentration (method no. 990.03; [28]) was analyzed via combustion using a nitrogen/protein determinator (LECO® FP-528, St. Joseph, MI, USA).

For the liquid effluent, concentrations of VFAs were analyzed using gas chromatography based on the method by Wang et al. [30], while the concentration of NH_3-N was determined following the procedure described by Broderick and Kang [23].

2.3.6. Calculations

Dry Matter Disappearance (DMD)

$$DMD\ (\%) = \frac{Initial\ feed\ weight - Residual\ feed\ weight}{Initial\ feed\ weight} \times 100 \quad (3)$$

Neutral Detergent Fiber, Starch, and N Disappearance

$$Disappearance\ (\%) = \frac{Initial\ content - Residual\ content}{Initial\ content} \times 100 \quad (4)$$

Volatile Fatty Acid (VFA) and Ammonia (NH_3-N) Production Rates

$$Production\ rate = Concentration\ (\mathrm{mmol/L}) \times Effluent\ volume\ (\mathrm{L/day}) \quad (5)$$

Gas Production and Methane Analysis

Gas Volume (L/day): Gas volume was measured daily using a flowmeter and averaged over the sampling period (days 8–10).

Methane Concentration (mg/m^3)

$$Concentration\ \left(\mathrm{mg/m^3}\right) = 0.0409 \times Concentration\ (\mathrm{ppm}) \times Molecularweight\ of\ CH_4 \quad (6)$$

Microbial Protein Synthesis Efficiency (g Bacteria/OM Digested)

$$Efficiency = \frac{Microbial\ protein\ (\mathrm{g})}{Digestible\ organic\ matter\ (\mathrm{g})} \quad (7)$$

2.4. Statistical Analysis

Only the data from the last 3 days of experiment were considered for statistical evaluation and were analyzed as a completely randomized design using 3 treatments, with a fermenter as the experimental unit. Data were tested for normality and submitted to analyses of variance with a 5% significance level. The MIXED procedure of SAS (SAS Inst., Inc., Cary, NC, USA) was used for all statistical analysis and inferences. All tables present least square means along with the standard error of the mean (SEM). The statistical model was as follows:

$$Yijkl = \mu + Ti + \varepsilon ijkl \quad (8)$$

where μ = general mean; Ti = fixed effect of the treatment I, for which linear and quadratic contrasts were tested; and εijkl = random error with a mean of zero and variance of σ^2, the variance among measures among fermenters. Linear and quadratic contrasts were tested whenever a statistical significance for the treatment effect was observed.

3. Results

The dataset suggested that altering the N content in saliva, either by a 15% or 30% reduction, did not differ significantly the pH values ($p > 0.05$) and NH_3-N ($p > 0.05$) outflow in the artificial rumen system. However, NH_3 concentrations within the fermenter followed a quadratic pattern, reaching their lowest point with the saliva that contained 30% less nitrogen ($p = 0.070$). The analysis of in vitro digestibility parameters showed that reducing N levels in saliva did not result in significant differences in the digestibility of DM, CP, NDF, or starch ($p > 0.05$). However, OM digestibility showed a quadratic trend with an increase at the 30% decrease in saliva N content ($p = 0.079$). The analysis of gas production parameters showed that altering N levels in saliva did not significantly alter either total gas production or CH_4 production in the Rusitec® system ($p > 0.05$). Similarly, the analysis

of VFA production demonstrated that alterations in saliva N levels did not significantly impact the production of acetate, propionate, butyrate, or total VFA ($p > 0.05$; Table 2). Lastly, the analysis revealed that altering N levels in saliva did not lead to significant differences in most microbial protein synthesis parameters. Only g Bac/OM dig ($p = 0.028$) and N flow ($p = 0.002$) showed a quadratic effect, both of which exhibited a reduction at the 30% decrease in saliva N content (Table 3).

Table 2. Effects of different nitrogen levels in artificial saliva on fermentation parameters, in vitro digestibility, nutrient composition, gas production, and volatile fatty acids profile.

Item	Treatment			SEM [1]	*p*-Value [2]	
	Control	−15	−30		L	Q
Fermentation parameter						
pH	6.71	6.80	6.81	0.040	0.161	0.337
NH_3-N Fermenter [3], mmol	21.23	19.57	16.85	1.4182	0.451	0.070
NH_3-N Outflow [4], mmol	19.55	18.21	19.64	2.179	0.685	0.783
In vitro digestibility, g						
Dry matter	59.06	56.62	60.75	1.721	0.292	0.128
Organic matter	58.81	58.19	62.31	1.164	0.798	0.079
Crude protein	46.68	42.75	47.43	2.561	0.319	0.408
Neutral detergent fiber	24.26	21.74	27.06	2.510	0.451	0.144
Starch	92.63	92.13	93.81	0.960	0.731	0.252
Gas production						
Total, L/d	0.186	0.189	0.168	0.017	0.919	0.399
Methane, mg/d	2.95	4.36	4.69	1.085	0.465	0.483
Volatile fatty acids production, mmol						
Acetate	827.58	962.51	1338.27	218.19	0.702	0.135
Propionate	444.81	361.59	545.05	64.045	0.426	0.105
Butyrate	326.18	259.27	474.69	96.678	0.668	0.163
Total	1598.57	1583.37	2358.00	348.37	0.978	0.107
Volatile fatty acids production, %						
Acetate	61.73	64.67	63.27	2.145	0.370	0.979
Propionate	21.03	19.98	20.56	0.566	0.232	0.936
Butyrate	17.24	15.35	16.17	2.450	0.610	0.967

[1] SEM = standard error of the mean. [2] L = linear effect; Q = quadratic effect. [3] NH_3 fermenter, mmol = ammonia nitrogen in the fermenter. [4] NH_3 outflow, mmol = ammonia nitrogen in the outflow.

Table 3. Effects of different nitrogen levels in artificial saliva on microbial protein synthesis, nitrogen digestion, utilization efficiency, and nitrogen flow dynamics.

Item	Treatment			SEM [1]	*p*-Value [2]	
	Control	−15	−30		L	Q
Microbial protein synthesis						
NH_3-N, g/d [3]	0.11	0.12	0.12	0.024	0.805	0.856
NAN, g/d [4]	0.33	0.30	0.30	0.028	0.396	0.556
Bacterial N, g/g [5]	0.25	0.23	0.19	0.024	0.640	0.111
NANMN, g/d [6]	0.09	0.07	0.09	0.012	0.247	0.191
N dig	0.83	0.81	0.83	0.014	0.325	0.349

Table 3. *Cont.*

Item	Treatment			SEM [1]	*p*-Value [2]	
	Control	−15	−30		L	Q
N use efficiency [7]	0.54	0.54	0.45	0.053	0.977	0.158
g Bac/OM dig, g/d [8]	34.93	32.01	24.32	3.003	0.523	0.028
RUP flow [9]	0.34	0.31	0.33	0.022	0.355	0.847
N flow [10]	0.58	0.54	0.51	0.007	0.006	0.002
RDP flow [11]	0.21	0.22	0.18	0.021	0.754	0.223

[1] SEM = standard error of the mean. [2] L = linear effect; Q = quadratic effect. [3] NH_3-N, g/d = ammonia nitrogen. [4] NAN, g/d = non-ammonia nitrogen. [5] Bacterial N, g/g = bacterial nitrogen. [6] NANMN, g/d = non-ammonia non-microbial nitrogen. [7] N use efficiency = nitrogen use efficiency. [8] g Bac/OM dig, g/d = grams of bacteria per organic matter digested. [9] RUP flow = rumen undegradable protein flow. [10] N flow = nitrogen flow. [11] RDP flow = rumen degradable protein flow.

4. Discussion

This study aimed to evaluate the impact of varying N levels in artificial saliva on ruminal fermentation parameters, microbial populations, and diet digestion in dairy cows using the Rusitec® system. Our hypothesis that reduced N would not affect fermentation was confirmed, except for microbial protein synthesis at the highest reduction level. As demonstrated by Capelari et al. [20], the Rusitec® method, initially developed to assess fibrous feed [16], has proven versatile for a range of applications. It has been effectively employed with mixed diets containing different starch levels [31,32], for evaluating the effects of feed additives on rumen metabolism [33], and for devising strategies to reduce methane emissions [34,35]. However, as dietary practices and herd management techniques evolve, it is essential to reassess the relevance of existing protocols. In this context, a reevaluation of McDougall's [10] pioneering artificial saliva formulation considering modern dairy cattle diets is needed. Such an evaluation would not only ensure the effectiveness of artificial saliva but also underscore the importance of refining methods for assessing dietary strategies to meet the dynamic demands of modern dairy farming practices.

Consistent with the use of the same diet, identical inoculum volume, and equal nutrient supply across all treatments, no significant differences were observed in VFA production. This contrasts with the observations made by Oss et al. [11], where a linear increase in total VFA production and a quadratic increase in valerate, isovalerate, and isobutyrate production were noted with increasing proportions of bison inoculum. Such variations were likely due to the linear increase in CP degradation (N disappearance) and the catabolism of branched-chain amino acids, as noted by Lindsay and Reynolds [36]. The concentration of VFAs in the rumen is influenced by various factors, including the rate and extent of OM digestion [15]. Highly digestible feeds tend to be broken down in the rumen, leading to VFA production as a byproduct [37]. The molar proportion of butyrate typically remains stable, usually ranging between 10 and 20%. An increase in butyrate concentration has been identified in animals on high-grain diets [38,39]. Ruminal metabolism of lactate can produce acetate, propionate, butyrate, and, to a lesser extent, caproic and valerate [40,41]. However, the dominant product can vary depending on the ruminal pH; under acidic conditions, butyrate is more likely to be produced from acetate [42,43]. It has been suggested that butyrate can be synthesized from acetate using the two hydrogen atoms released during the oxidation of lactate to pyruvate, which could make butyrate production a potential hydrogen sink [44,45]. These findings suggest that, despite changes in N levels, the stable production of acetate, propionate, and butyrate across treatments may be associated with the maintenance of steady pH conditions in the fermenters.

In addition to the VFA results, the lack of effect on total gas and CH_4 production aligns with the absence of significant changes in DM and CP degradation, corroborating the lack of variations observed in the acetate–propionate ratio. This outcome is somewhat expected, especially considering that an increase in the acetate–propionate ratio typically corresponds with increased CH_4 production [46,47]. Interestingly, although the reduced N levels in the saliva did not directly affect nutrient digestibility or VFA concentrations, the observed decrease in N flow reflects the reduced N availability in the rumen, directly impacting the EMPS. Thus, while part of our hypothesis was confirmed—fermentation remained stable with reduced N—the decline in EMPS at the highest level of N reduction suggests a threshold below which microbial activity may be compromised.

The study by Brandao et al. [15] demonstrated that increasing dietary non-fibrous carbohydrate (NFC) concentration led to a negative linear response in NH_3-N levels, likely due to enhanced microbial protein synthesis, which reduces NH_3-N accumulation [48,49]. Additionally, bacterial N as a proportion of total N increased with higher dietary NFC levels, while the proportion of NANMN to total N remained unaffected by NFC concentration. In the present study, despite using the same diet (and thus the same NFC levels) across all treatments, microbial protein synthesis (g Bac/OM dig) exhibited a significant quadratic effect, with the lowest value observed at the highest treatment level. This indicates that microbial protein yield varied across treatments, reinforcing the need to assess how EMPS responds beyond NFC availability. However, direct measures of EMPS, reflected by the consistent N use efficiency and N digestibility, remained unchanged. This stability in EMPS suggests that although total microbial protein output (g Bac/OM dig) varied, the relative efficiency of N utilization by microbes did not. Although previous studies have shown that bacterial N flow decreases as ruminal pH rises, EMPS is not directly linked to rumen pH, which typically fluctuates with carbohydrate fermentability [50]. Moreover, ruminal NH_3-N was found to be insensitive to EMPS [15], aligning with findings from Bach et al. [50] and Oba and Allen [48]. In contrast, our study observed that most microbial protein synthesis parameters showed no significant variation except for g Bac/OM dig and N flow. This trend suggests a shift in microbial N utilization dynamics may explain the quadratic decrease EMPS observed at the lowest N levels. The decrease in N flow, driven by reduced N availability in artificial saliva, likely increases energy availability for microbial communities, altering microbial protein flow. Thus, while microbial activity remains stable, the efficiency of converting available energy into microbial protein is impacted, potentially due to imbalances in the ruminal nitrogen-to-energy ratio.

Lastly, it is important to acknowledge a limitation of this study: the sample size was relatively small, with six fermenters per treatment, constrained by the equipment's capacity. Conducting a more comprehensive experiment with a larger sample size would increase statistical power, potentially revealing results that might have been missed in the present study. One way to achieve this is by performing multiple rounds of the trial and combining the data into a larger dataset, thereby increasing the robustness and depth of the findings. This approach would enhance our understanding of the observed trends and provide more insights, leading to more conclusive results.

5. Conclusions

The findings from this study demonstrate that reducing N in artificial saliva by up to 15% is consistent with the initial hypothesis, maintaining fermentation and microbial protein synthesis, but further reductions (30%) may impair microbial protein yield, partially rejecting the hypothesis. Reducing nitrogen levels in artificial saliva by up to 30% did not significantly affect ruminal pH, ammonia nitrogen concentrations, or ammonia nitrogen outflow in the Rusitec® system, indicating that nitrogen reduction maintains ruminal

stability. Additionally, digestibility of nutrients (dry matter, crude protein, neutral detergent fiber, and starch), gas production and methane production, and volatile fatty acid profiles remained consistent across treatments, demonstrating that lowering nitrogen levels in the artificial saliva does not compromise fermentation efficiency or dietary digestion. These findings highlight the potential for reducing nitrogen in artificial saliva without impairing ruminal function, providing a foundation for future research to refine nitrogen dynamics in in vitro systems and better simulate modern dairy cow diets.

Author Contributions: Conceptualization, M.I.M.; methodology, Í.R.R.d.C., L.d.N.C.d.S., G.B.C.L., I.F.C., E.M.d.P., A.M.C. and M.I.M.; software, M.I.M.; validation, Í.R.R.d.C., E.M.d.P. and M.I.M.; formal analysis, Í.R.R.d.C., L.d.N.C.d.S., I.F.C. and M.I.M.; investigation, Í.R.R.d.C.; resources, M.I.M.; data curation, Í.R.R.d.C., E.M.d.P. and M.I.M.; writing—original draft preparation, Í.R.R.d.C.; writing—review and editing, Í.R.R.d.C., L.d.N.C.d.S., G.B.C.L., I.F.C., E.M.d.P., A.M.C. and M.I.M.; visualization, M.I.M.; supervision, M.I.M.; project administration, Í.R.R.d.C., G.B.C.L., A.M.C. and M.I.M.; funding acquisition, M.I.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The ruminal content of donor animals utilized in this study were handled by the guidelines set by Washington State's Institutional Animal Care and Use Committee (WSU/IACUC; ASAF #7723; 13 February 2025).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: The first author thanks the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) and Washington State University for their assistance in funding his PhD research. During the preparation of this manuscript/study, no GenAI has been used for purposes such as generating text, data, or graphics, or for study design, data collection, analysis, or interpretation of data.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

N	Nitrogen
NH_3-N	Ammonia Nitrogen
CP	Crude Protein
TMR	Total Mixed Ratio
DM	Dry Matter
NDF	Neutral Detergent Fiber
VFA	Volatile Fatty Acids
CH_4	Methane
OM	Organic Matter
NFC	Non-Fibrous Carbohydrate
EMPS	Efficiency of Microbial Protein Synthesis
RUP	Rumen Undegradable Protein
RDP	Rumen Degradable Protein
NAN	Non-Ammonia Nitrogen
NANMN	Non-Ammonia Non-Microbial Nitrogen
DMD	Dry Matter Disappearance
GC	Gas Chromatograph

FID	Flame Ionization Detector
WS	Wind Speed
AOAC	Association of Official Analytical Chemists
SEM	Standard Error of the Mean
L	Linear Effect
Q	Quadratic Effect
WSU/IACUC	Washington State University Institutional Animal Care and Use Committee
ID	Inner Diameter
OD	Outer Diameter
N_2	Nitrogen Gas
O_2	Oxygen
H_2SO_4	Sulfuric Acid
^{15}N	Nitrogen-15 (isotope)
NH_4	Ammonium
SO_4	Sulfate
NaCl	Sodium Chloride
H	Hydrogen
Ar	Argon
CO_2	Carbon Dioxide
ppm	Parts per Million
mg/m^3	Milligrams per Cubic Meter
kPa	Kilopascal
mL/min	Milliliters per Minute
L/d	Liters per Day
g/d	Grams per Day
mmol/L	Millimoles per Liter
mmol/d	Millimoles per Day
AU	Area Unit
R	Gas Constant
°K	Kelvin

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