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Effectiveness of Diets Incorporating Nano-Zeolite and Urea-Impregnated Nano-Zeolite on Fermentation Efficiency in Rumen Fluid *In Vitro*

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ABSTRACT

Zeolite had been widely investigated in animal feed for its ability to improve feed efficiency and reduce ammonia emissions, and nano-zeolite offered greater adsorption capacity due to its expanded surface area. This study evaluated the effects of nano-zeolite supplementation on ammonia (NH_3), volatile fatty acids (VFA), the VFA/NH_3 ratio, and pH during in vitro rumen fermentation. A two-factor randomized block design (6 × 6) with four replicates was used. Incubation time (2, 4, 8, 12, 24, and 48 h) served as the first factor, and six dietary treatments served as the second factor: control, urea-supplemented concentrate, inactivated nano-zeolite, activated nano-zeolite, urea-impregnated inactivated nano-zeolite, and urea-impregnated activated nano-zeolite. Nano-zeolite, whether active or urea-impregnated, did not significantly improve rumen fermentation efficiency based on NH_3, VFA, or VFA/NH_3 ratio. All nano-zeolite diets performed similarly to the control and urea-only diets. Fermentation efficiency peaked at 4 and 8 hours of incubation, indicating that nano-zeolite combined with urea supported stable fermentation, particularly during early to mid-incubation.

1. Introduction

Tropical forages are widely characterized by high structural fiber content and low soluble carbohydrate and nitrogen fractions, which collectively constrain microbial degradation in the rumen (Van Soest, 2018; Tedeschi et al., 2019). Under such conditions, the rate of carbohydrate fermentation slows, and microbial protein synthesis becomes limited by asynchronous availability of fermentable energy and nitrogen (Haque et al., 2019). Synchronizing the release of nitrogen with carbohydrate degradation is therefore essential to improve fermentation efficiency and reduce nitrogen losses (Kardaya et al., 2023). When nitrogen becomes available too rapidly—such as from conventional urea—rumen microbes cannot capture it efficiently, leading to excess ammonia accumulation and reduced feed utilization (Patra, 2016).

Zeolite has been widely studied as a feed additive capable of modifying rumen fermentation by adsorbing ammonia, buffering pH, and improving nutrient utilization (Wang et al., 2025; Kozłowski et al., 2020). Kardaya et al. (2009) first advanced the concept of urea-impregnated zeolite (UZ) as a *slow-release urea (SRU)* agent. This approach enabled a controlled release of ammonia during in vitro fermentation, reducing nitrogen losses and stabilizing pH. Subsequent vivo research further confirmed that UZ supplementation in sheep improved fermentation patterns by lowering ammonia and acetate concentrations while enhancing propionate production and maintaining normal plasma urea levels (Kardaya et al., 2011; Nguyen & Dang, 2018).

Recent advances in nanotechnology have opened new possibilities for enhancing the functional properties of mineral-based feed additives. Nanomaterials possess extremely large surface areas, high porosity, and enhanced ion-exchange capacity, which can alter chemical reactivity and adsorption characteristics within the rumen environment (Duhan et al., 2017). In livestock systems, nanotechnology applications—often referred to as *agro-nanotechnology*—have shown promise for improving nutrient delivery, reducing environmental emissions, and enhancing feed efficiency (El-Nile et al., 2021; El-Saadony et al., 2023). When zeolite is processed to the nanoscale, its adsorption capacity for cations, including ammonium, increases markedly compared to the conventional form (Kardaya et al., 2025).

Despite the known benefits of zeolite, studies specifically evaluating nano-zeolite in rumen fermentation remain limited. El-Nile et al. (2021) reported that nano-zeolite improved the rumen fermentation pattern and nutrient digestibility in ruminants; however, results have varied across diet types and experimental conditions. The nanoscale structure enables more efficient adsorption of urea and ammonia, raising the possibility that nano-zeolite could serve as an improved alternative to conventional slow-release urea technologies (Mousa et al., 2022). Moreover, nano-zeolite may influence fermentation by supporting microbial growth, stabilizing rumen pH, and reducing nitrogen waste through enhanced sequestration of free ammonia (Arshad et al., 2023).

Given the diverse capabilities of nano-zeolites, there is an expectation that incorporating them into diets could enhance the efficiency of rumen fermentation while mitigating its adverse effects. Consequently, it is essential to perform in vitro experiments to assess ammonia (NH3) levels, Volatile Fatty Acid (VFA) concentrations, and pH levels, which are crucial indicators of microbial fermentation efficiency in the rumen. This research seeks to evaluate how nano-zeolites in diets influence the production of ammonia (NH3), VFA, and the pH of rumen fluid in vitro.

2. Methods

The National Research and Innovation Agency's Ethics Commission for Animal Maintenance and Use has granted its approval and authorization for all procedures in this study, following the Research Ethics Clearance in the Field of Livestock and Utilization, with reference number 203/KE.02/SK/08/2024.

The ingredients used consisted of six types of experimental diets, rumen liquid, CO_2 gas, and saturated $HgCl_2$, $NaHCO_3$ 9.80 g/L, *Anhydrous* Na_2HPO_4 3.71 g/L, KCl 0.57 g/L, NaCl 0.47 g/L, *Heptahydrate* $MgSO_4$ 0.12 g/L, *Dihydrate* $CaCl_2$ 0.05 g/L, and urea (*reagent grade*) 0.90 g/L, supernatant, Na_2CO_3 saturated, HCl 0.01 N, mixed indicator solution, boric acid solution, H_{2SO4} 15%, supernatants, NaOH 0.5 N, aquadest, PP indicator 1%, and HCl 0.5 N. Table 1 displays the composition of the feed ingredients included in the diet, while Table 2 outlines the nutrient content.

Table 1 Feed ingredients that make up the diet and nutrient content of the proximate analysis

Ingredients	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**
	Dry matter basis (%)					
Field Grass	55	55	55	55	55	55
Pollard	4	11	2	2	6.6	6.6
Yellow Corn	14	15	15	15	16	16
Soybean Meal	16.5	10.2	17	17	14	14
Coconut Meal	9.5	5	9	9	4.8	4.8
Molasses	-	1	-	-	1	1
Inactive nano-zeolite	-	-	1	-	-	-
Activated nano-zeolite	-	-	-	1	-	-
Urea	-	0.8	-	-	-	-
Urea-impregnated inactive nano-zeolite	-	-	-	-	1.6	-
Urea-impregnated active nano-zeolite	-	-	-	-	-	1.6
Squirt	1	2	1	1	1	1
Total	100	100	100	100	100	100

Table 2 Nutrient contents of diets based on proximal analysis

Nutritional Content	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**
Dry Matter (%)	91.24	89.23	91.83	92.00	91.08	90.63
Crude Protein (%)	16.86	16.41	17.48	17.44	16.90	16.92
Crude Fat (%)	2.11	2.05	2.00	1.89	1.98	1.90
Crude Fiber (%)	19.72	19.51	19.78	19.55	19.42	19.34
Ash (%)	9.98	9.81	10.92	10.86	10.74	10.68
NFE (%) *	42.57	41.45	41.66	45.26	42.04	41.79
DE (Kcal/kg) *	3275	3243	3266	3216	3229	3210
ME (Kcal/kg) *	2686	2659	2678	2637	2648	2632

R1= Control diet without supplementation, R2= Urea-supplemented concentrate diet, R3= Inactivated nano-zeolite supplemented concentrate diet, R4= Active nano-zeolite supplemented concentrate diet, R5= Urea-impregnated inactive nano-zeolite supplemented concentrate diet, R6= Urea-impregnated active nano-zeolite supplemented concentrate diet. *) Calculation results: NFE (Nitrogen Free Extract) = %BK – (%PK+%LK+%SK+%Abu), DE (*Digestible Energy*); ME (Metabolizable Energy) = 0.82 x DE (NRC 2001).

2.1. Research Methods

This research employed a 2-factor Randomized Block Design (RBD) structured as 6 x 6 with 4 repetitions. The first factor involved the incubation duration, which had 6 different time periods (2, 4, 8, 12, 24, and 48 hours), while the second factor was the experimental diet, also comprising 6 variations, resulting in a total of 168 experimental units. The experimental diet treatments included 6 types: a control diet without any supplementation (R1), a concentrated diet with added urea (R2), a concentrated diet with inactivated nano-zeolite (R3), a concentrated diet with active nano-zeolite (R4), a concentrated diet with inactivated nano-zeolite combined with urea (R5), and a concentrated diet with active nano-zeolite combined with urea (R6). The study focused on observing variables such as ammonia production (NH3), VFA, the VFA:NH3 ratio, and pH levels.

The collected data were subjected to multivariate General Linear Model (GLM) analysis using the IBM SPSS 27 software on the variables studied. If a significant difference was detected ($P<0.05$), a subsequent analysis was performed using the Duncan Multiple Range Test.

2.2. Research Procedure

2.2.1. Preparation of Nano-zeolite and Urea Impregnated Nano-zeolite

The process of creating urea-impregnated nano-zeolite begins with the preparation of natural zeolite, which is ground into a fine powder with a mesh size of 200. Zeolites are divided into two categories: inactive and those activated at 600 °C. To make urea-impregnated zeolite, 310 grams of urea are dissolved in distilled water at temperatures between 80 and 119 °C. Once the urea is dissolved, zeolite is added to the mixture and stirred thoroughly until it cools, after which it is ground again. After separating the zeolite based on treatment, its size is further reduced using nanotechnology. These processed nano-zeolites are then incorporated into the diet.

2.2.2. Sample Preparation

The sample was composed of a blend of 55 g of forage and 45 g of concentrate, measured on a dry matter basis, which were then mashed together. A quantity of 0.5 g of these samples was placed into a 100 ml glass bottle, labeled according to the test and sample treatment.

2.2.3. Intake of Rumen Fluid

Rumen fluid is obtained from fistulated cattle housed at the BRIN Cibinong cattle barn. To begin, a thermos is preheated with water at 39°C, which is then discarded before the thermos is filled with rumen fluid to maintain microbial viability. The collection process involves sampling from different areas within the rumen, capturing both solid and liquid parts). The thermos containing the rumen fluid is then taken to the laboratory, where CO_2 is promptly introduced. CO_2 gas is infused into the rumen liquid inside the thermos, and the contents are mixed to ensure the solid and liquid rumen inoculum are uniform, followed by filtration through two layers of gauze. CO_2 gas is continuously added for 30 seconds throughout the procedure (Camacho *et al.,* 2019) .

2.2.4. Preparation of McDougall Buffer Solution

McDougall's buffer solution is composed of sodium bicarbonate, which acts as a buffer. McDougall buffers were selected due to their ability to create a more favorable environment for fiber digestion (Camacho *et al.,* 2019). The materials for the buffer solution are measured and placed into an Erlenmeyer flask. Then, 1.5 L of distilled water is added, and the mixture is homogenized using a magnetic stirrer. The $CaCl_2.2H_2O$ is introduced at the end, once all other components are fully dissolved. After thorough mixing, CO_2 gas is passed through the solution until the pH reaches 6.8.

Following the methods described by Menke (1988), the incubation media solution is created by mixing one portion of the filtered rumen fluid with two portions of McDougall's solution. Table 3 outlines a timetable for incubation periods along with the respective amounts of the incubation media solution mixture.

Table 3 Incubation time and amount of incubation media solution mixture

Incubation period (hours)	**Sample (6R,4U)**	**Rumen fluid (mL)**	**Buffer (mL)**	**Total rumen fluid + buffer (mL)**
		Ratio 1:2		
2	7 x 6	700	1400	2100
4	7 x 6	700	1400	2100
8	7 x 6	700	1400	2100
12	7 x 6	700	1400	2100
24	7 x 6	700	1400	2100
48	7 x 6	700	1400	2100
Total	252	4.200	8.400	12.600

In each glass bottle, 0.5 g of the sample was combined with 50 ml of incubation media solution, followed by a 30-second infusion of CO_2 gas. The bottles were then sealed with a rubber stopper and an aluminum seal using a vial crimper. These bottles were placed in a shaking water bath at 39°C for incubation periods of 2 (I2), 4 (I4), 8 (I8), 12 (I12), 24 (I24), and 48 (I48) hours. Following each incubation period, the sample in the tube is mixed thoroughly using a vortex, its pH is measured, and 2 to 3 drops of saturated $HgCl_2$ are added to halt the fermentation process. Subsequently, it is placed in the freezer for VFA and NH3 analysis.

2.2.5. Ammonia Analysis (NH_3)

Ammonia (NH_3) was examined using the Conway microdiffusion technique. In this process, 1 ml of a saturated sodium carbonate (Na_2CO_3) solution is placed on one side of the cup's partition, while 1 ml of the incubation supernatant is added to the opposite side. The center of the cup is filled with 1 ml of boric acid, which contains the indicators bromocresol green and methyl red (BCG: MR). The cup is then securely sealed with a lid coated with vaseline. The saturated Na_2CO_3solution and the supernatant are combined by shaking the cup, and the mixture is left undisturbed for 24 hours at room temperature. During this period, the boric acid solution's color shifts from red to a purple-bluish hue. After 24 hours, the sample undergoes titration with a 0.01 N HCl solution until the solution turns bright red. The NH_3 concentration is determined using the formula:

$$\text{N-NH}_3\ (\text{mM}) = \frac{(\text{Va}-\text{Vb})\ \text{x N HCL x } 1000}{\text{Vc}}$$

Va = Volume of sample titration
Vb = volume of blank titration
Vc = Sample volume
N HCl = Normality of HCL

2.2.6. VFA Analysis

Volatile fatty acids (VFA) can be measured using a modified version of the steam distillation method outlined in the General Laboratory Procedure (1966). The distillation equipment, Omni Lab Food Alyt, is used, and the D3000 is activated to allow the steam generator to heat up. A 5 ml supernatant sample is placed into a VFA distillation tube, followed by the addition of 1 ml of 15% H_2SO_4. The distillation tube is securely connected to the distiller. The distillate is collected in a 250 ml Erlenmeyer flask that already contains 5 ml of a 0.5 N NaOH solution. The distillation is halted once the distillate volume reaches 100 ml.

Two drops of a 1% phenolphthalein (PP) indicator are added to the Erlenmeyer flask with the distillate. Titration is performed using a 0.5 N HCl solution until the solution changes from pink to colorless. The same procedure is followed for blanks as in VFA measurements, but without using supernatants from the experimental sample. The VFA concentration is determined using the formula:

$$\text{Total VFA (mM)} = \frac{(\text{v. titrant blank} - \text{v. titrant sample}) \times \text{N HCl} \times 1000}{\text{v. sampel}}$$

N-HCl = Normality of HCl solution
v. titrant blank = Quantity of HCl needed to neutralize 5 ml of NaOH (blank)
v. titrant sample = Quantity of HCl titters required to condense the distillation outcome

3. Results and Discussion

3.1. Results

3.1.1. Ammonia Production

Ammonia generation serves as a useful measure for evaluating the fermentability of feed, which is linked to the digestibility of feed protein, as well as the activity and population of rumen microbes. Monitoring the ammonia concentration in the rumen is crucial, as it significantly influences the optimal growth of rumen microorganisms. Approximately 80% of these microbes can utilize ammonia as a nitrogen source for their development. Table 4 below presents the ammonia production levels across different dietary treatments during the incubation period.

Table 4 Concentrations of ammonia produced under different dietary treatments during the incubation phase

Hour	Diets						
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	**Means**
2	12.37±2.41	12.92±2.65	11.70±2.72	12.25±2.83	12.25±3.36	13.25±1.50	12.45±2.39[a]
4	12.20±2.08	12.67±4.33	11.95±2.49	11.57±2.93	11.42±5.41	11.00±5.19	11.80±3.55[a]
8	12.02±3.46	11.07±4.60	11.12±4.69	12.02±2.99	13.37±2.01	11.47±3.70	11.85±3.36[a]
12	14.73±0.73	13.83±1.76	14.13±0.58	14.03±0.35	14.90±0.43	14.33±0.50	14.32±0.83[b]
24	14.42±4.06	14.67±2.74	15.05±2.15	14.55±3.10	14.42±2.17	15.92±1.52	14.84±2.49[b]
48	16.32±2.91	18.82±1.60	16.87±3.25	17.70±5.14	17.45±3.67	19.80±0.80	17.82±3.09[c]

R1= Control diet without supplementation, R2= Urea-supplemented concentrate diet, R3= Inactivated nano-zeolite supplemented concentrate diet, R4= Active nano-zeolite supplemented concentrate diet, R5= Urea-impregnated inactive nano-zeolite supplemented concentrate diet, R6= Urea-impregnated active nano-zeolite supplemented concentrate diet. Different superscripts in the same column show a marked difference ($P<0.05$) based on the results of Duncan's analysis.

3.1.2. Production of Volatile Fatty Acids (VFA)

The in vitro VFA analysis results (Table 5) indicated differences in concentration depending on the incubation times and diet treatments.

Table 5 VFA Concentration (mM) Across Different Diet Treatments Over Incubation Period

Hour	Diets						
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	**Means**
2	114.79±23.89	111.57±29.00	113.25±37.95	97.57±20.28	98.83±19.97	101.81±21.84	106.30±24.33[a]
4	107.14±18.80	105.78±13.99	101.75±18.46	107.77±16.77	107.04±13.17	96.95±19.48	104.40±15.51[a]
8	105.83±18.09	100.73±17.26	110.68±19.15	110.17±21.67	109.07±12.84	107.57±16.76	107.34±16.14[a]
12	128.95±8.80	107.99±30.25	106.95±27.91	105.40±36.37	101.56±28.83	100.18±28.46	108.51±25.57[a]
24	127.78±13.50	121.94±13.12	128.22±9.83	128.06±6.00	122.48±7.06	134.50±10.37	127.16±10.12[b]

48	123.33±22.38	125.69±17.86	128.77±24.81	124.52±24.13	120.81±18.95	117.00±19.88	123.35±19.39[b]

Remark: R1= Control diet without supplementation, R2= Urea-supplemented concentrate diet, R3= Inactivated nano-zeolite supplemented concentrate diet, R4= Active nano-zeolite supplemented concentrate diet, R5= Urea-impregnated inactive nano-zeolite supplemented concentrate diet, R6= Urea-impregnated active nano-zeolite supplemented concentrate diet. The different superscripts in the same column show a marked difference (P<0.05) based on the results of Duncan's analysis

3.1.3. Ratio VFA/NH_3

Table 6 below displays the data concerning the ratio of volatile fatty acids (VFA) to ammonia (NH_3) derived from in vitro fermentation outcomes across different feed treatments.

Table 6 Ratio of VFA to NH_3 Across Different Dietary Treatments Over Incubation Period

Hour	Diets						
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	**Means**
2	9.49±2.31	9.09±3.41	10.33±4.54	8.32±2.52	8.76±3.55	7.88±2.44	8.98±2.97[ab]
4	9.15±3.15	9.38±4.08	8.86±2.63	9.98±3.61	12.65±9.26	12.19±9.73	10.37±5.65[b]
8	9.53±3.62	10.35±4.07	12.48±8.08	9.77±3.61	8.36±2.10	10.45±4.59	10.16±4.36[b]
12	8.75±0.54	8.09±3.43	7.61±2.23	7.47±2.38	6.78±1.71	7.04±2.25	7.62±2.01[a]
24	9.40±2.65	8.45±1.19	8.63±1.26	9.23±2.70	8.65±1.54	8.51±1.21	8.81±1.70[ab]
48	7.60±0.86	6.68±0.86	7.69±0.98	7.31±1.50	7.05±1.06	5.93±1.20	7.04±1.15[a]

Description: R1= Control diet without supplementation, R2= Urea-supplemented concentrate diet, R3= Inactivated nano-zeolite-supplemented concentrate diet, R4= Active nano-zeolite-supplemented concentrate diet, R5= Urea-impregnated inactive nano-zeolite supplemented concentrate diet, R6= Urea-impregnated active nano-zeolite supplemented concentrate diet. The different superscripts in the same column show a marked difference (P<0.05) based on the results of Duncan's analysis

3.1.4. pH Level

Table 7 displays the pH assessment data for fermentation media across different dietary treatments (R1 to R6) during the incubation periods I2, I4, I8, I12, I24, and I48.

Table 7 Rumen Fluid pH Levels Across Different Dietary Treatments During the Incubation Phase

Hour	Diets						
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	**Means**
2	7.09±0.07	7.13±0.13	7.13±0.13	7.13±0.02	7.13±0.05	7.14±0.05	7.13±0.08[c]
4	7.06±0.10	7.06±0.08	7.04±0.07	7.08±0.76	7.09±0.08	7.10±0.09	7.07±0.07[c]
8	7.00±0.11	6.98±0.16	7.01±0.19	7.01±0.10	7.02±0.13	7.00±0.13	7.00±0.12[b]
12	6.95±0.09	6.98±0.12	6.96±0.11	6.97±0.10	6.99±0.14	6.98±0.13	6.97±0.10[b]
24	6.86±0.09	6.86±0.09	6.88±0.07	6.88±0.03	6.88±0.09	6.92±0.11	6.88±0.08[a]
48	6.83±0.08	6.85±0.08	6.84±0.06	6.86±0.07	6.82±0.08	6.86±0.09	6.84±0.07[a]

Description: R1= Control diet without supplementation, R2= Urea-supplemented concentrate diet, R3= Inactivated nano-zeolite-supplemented concentrate diet, R4= Active nano-zeolite-supplemented concentrate diet, R5= Urea-impregnated inactive nano-zeolite supplemented concentrate diet, R6= Urea-impregnated active nano-zeolite supplemented concentrate diet. The different superscripts in the same column show a marked difference (P<0.05) based on the results of Duncan's analysis

3.2. Discussion

3.2.1. Ammonia Production

Overall, ammonia levels for each treatment diet increased from incubation I2 to I48, with values ranging from 11.7 mM in R3 to 19.8 mM in R6. The ammonia concentrations reported by Kardaya *et al.* (2009) ranged from 7.5 mM to 22.45 mM, which closely align with

the findings of this study. Similarly, Kardaya *et al.* (2025) observed ammonia concentrations between 8.11 – 11.6 mM. The GLM multivariate full factorial analysis indicated no interaction between incubation duration (hours) and diet treatment. While diet treatment did not affect.

Table 8 Ratio of VFA to NH_3 Across Different Dietary Treatments Over Incubation Period

Hour	Diets						
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	**Means**
2	9.49±2.31	9.09±3.41	10.33±4.54	8.32±2.52	8.76±3.55	7.88±2.44	8.98±2.97^{ab}
4	9.15±3.15	9.38±4.08	8.86±2.63	9.98±3.61	12.65±9.26	12.19±9.73	10.37±5.65^{b}
8	9.53±3.62	10.35±4.07	12.48±8.08	9.77±3.61	8.36±2.10	10.45±4.59	10.16±4.36^{b}
12	8.75±0.54	8.09±3.43	7.61±2.23	7.47±2.38	6.78±1.71	7.04±2.25	7.62±2.01^{a}
24	9.40±2.65	8.45±1.19	8.63±1.26	9.23±2.70	8.65±1.54	8.51±1.21	8.81±1.70^{ab}
48	7.60±0.86	6.68±0.86	7.69±0.98	7.31±1.50	7.05±1.06	5.93±1.20	7.04±1.15^{a}

Description: R1= Control diet without supplementation, R2= Urea-supplemented concentrate diet, R3= Inactivated nano-zeolite-supplemented concentrate diet, R4= Active nano-zeolite-supplemented concentrate diet, R5= Urea-impregnated inactive nano-zeolite supplemented concentrate diet, R6= Urea-impregnated active nano-zeolite supplemented concentrate diet. The different superscripts in the same column show a marked difference (P<0.05) based on the results of Duncan's analysis

3.1.4. pH Level

Table 9 displays the pH assessment data for fermentation media across different dietary treatments (R1 to R6) during the incubation periods I2, I4, I8, I12, I24, and I48.

Table 9 Rumen Fluid pH Levels Across Different Dietary Treatments During the Incubation Phase

Hour	Diets						
	R1	**R2**	**R3**	**R4**	**R5**	**R6**	**Means**
2	7.09±0.07	7.13±0.13	7.13±0.13	7.13±0.02	7.13±0.05	7.14±0.05	7.13±0.08^{c}
4	7.06±0.10	7.06±0.08	7.04±0.07	7.08±0.76	7.09±0.08	7.10±0.09	7.07±0.07^{c}
8	7.00±0.11	6.98±0.16	7.01±0.19	7.01±0.10	7.02±0.13	7.00±0.13	7.00±0.12^{b}
12	6.95±0.09	6.98±0.12	6.96±0.11	6.97±0.10	6.99±0.14	6.98±0.13	6.97±0.10^{b}
24	6.86±0.09	6.86±0.09	6.88±0.07	6.88±0.03	6.88±0.09	6.92±0.11	6.88±0.08^{a}
48	6.83±0.08	6.85±0.08	6.84±0.06	6.86±0.07	6.82±0.08	6.86±0.09	6.84±0.07^{a}

Description: R1= Control diet without supplementation, R2= Urea-supplemented concentrate diet, R3= Inactivated nano-zeolite-supplemented concentrate diet, R4= Active nano-zeolite-supplemented concentrate diet, R5= Urea-impregnated inactive nano-zeolite supplemented concentrate diet, R6= Urea-impregnated active nano-zeolite supplemented concentrate diet. The different superscripts in the same column show a marked difference (P<0.05) based on the results of Duncan's analysis.

3.2. Discussion

3.2.1. Ammonia Production

Overall, ammonia levels for each treatment diet increased from incubation I2 to I48, with values ranging from 11.7 mM in R3 to 19.8 mM in R6. The ammonia concentrations reported by Kardaya *et al.* (2009) ranged from 7.5 mM to 22.45 mM, which closely align with the findings of this study. Similarly, Kardaya *et al.* (2025) observed ammonia concentrations between 8.11 – 11.6 mM. The GLM multivariate full factorial analysis indicated no interaction between incubation duration (hours) and diet treatment. While diet treatment did not affectammonia concentration, the incubation duration significantly influenced (P<0.05) ammonia levels in rumen fluids. Duncan Multiple Distance Analysis follow-up tests revealed that the lowest ammonia concentrations (P<0.05) occurred at incubation periods I2, I4, and I8

hours, while the highest (P<0.05) was at I48. These findings are consistent with Kardaya *et al.* (2009), who noted that a rice straw-based diet with slow-release urea and varying molasses levels increased rumen fluid ammonia concentrations at I2, decreased to a minimum at I8, and then peaked at I48. Prommachart *et al.* (2024) also found a similar increase in ammonia concentration at I8 using a diet of mangosteen, rambutan, and banana flower peel feed (MARABAC) with tung oil. Figure 1 illustrates the average ammonia concentration across different diet treatments during the incubation period.

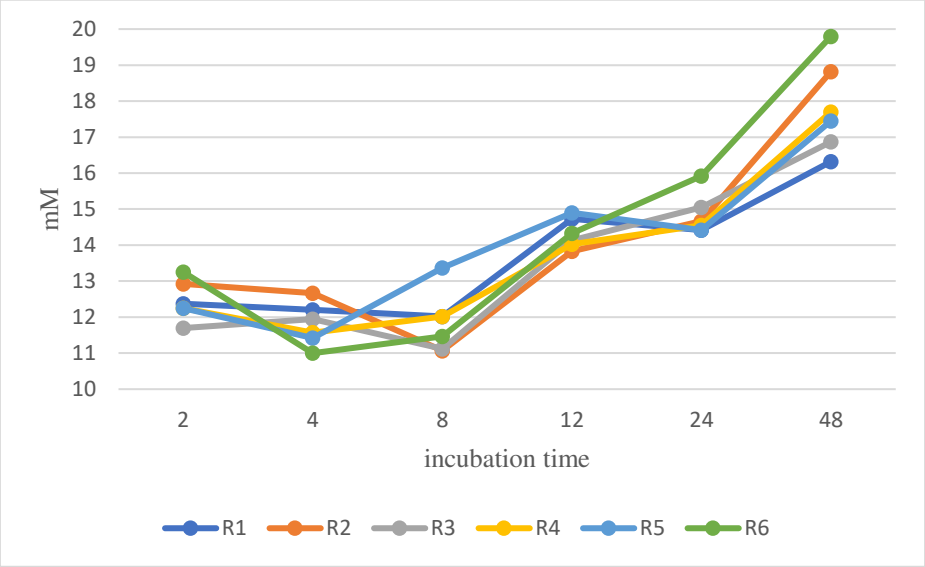

Figure 1 Mean concentration of ammonia production across different dietary treatments throughout the incubation period

Ammonia production levels are influenced by the crude protein content in animal feed. Protein sources that are readily broken down by rumen microbes are crucial in this process. Once feed proteins reach the rumen, they are decomposed by microbes into amino acids, which are subsequently deaminated to produce ammonia, organic acids, and CO_2. Not all proteins undergo degradation. The ammonia generated in the rumen combines with keto alpha acids derived from protein or carbohydrate sources to create new amino acids, which are essential for synthesizing microbial proteins (Hackman & Firkins (2015).

Detmann *et al.* (2014) also noted that as the amount of digestible organic matter rises, there is a corresponding linear increase in rumen ammonia nitrogen levels. This trend is at least partially attributed to the positive correlation between digestible organic matter in the diet and Crude Protein. The study's use of diets with relatively similar protein and energy levels is believed to contribute to the lack of significant treatment effects.

Referring to the data presented in Table 4, the R2 treatment with urea supplementation and the R6 treatment with active nano-zeolite supplementation exhibited the highest concentrations during the I2 incubation, measuring 12.92 mM and 13.25 mM, respectively. A reduction in NH_3 concentration was observed from hours I2 to I4 across all treatments (R1 to R6). The maximum NH_3 concentration in the cow's rumen is reached in the morning and evening, approximately 1 to 2 hours and 6 to 7 hours after the cow ingests the diet. This pattern is consistent for both 50:50 unbuffered, 50:50 buffered, 75:25 unbuffered, and 75:25 buffered diets (Khorasani and Kennelly 2001; Fathul and Wajizah, 2010).

From I4 to I8, NH_3 concentrations either continue to decrease or remain unchanged across most treatments. Specifically, the R1 treatment saw a reduction from 12.20 mM to 12.02 mM, whereas the R5 treatment experienced a slight increase from 11.42 mM to 13.37 mM. These findings align with the research by (Kardaya et al. 2025), which reported values between 8.1 and 11.6 mM. These range are conducive to protein metabolism in the rumen and

can enhance microbial protein synthesis.

Incubating all treatments from I8 to I12 led to a notable rise in NH_3 levels. Specifically, the R1 treatment saw an increase from 12.02 mM to 14.73 mM, while the R6 treatment rose from 11.47 mM to 14.33 mM. This can be attributed to the inclusion of zeolites in urea-based feeds, which enhances rumen fermentation and facilitates the absorption of NH_3-N generated from the breakdown of urea by the urease enzyme in the rumen, allowing for a controlled release as required. This process offers a consistent source for microbial protein synthesis by rumen microorganisms and helps maintain an optimal environment for their growth (Alrez *et al.,* 2024).

Ammonia concentrations from I12 to I48 during incubation led to an increase in NH3 levels across all treatments. The R2 treatment, with 18.82 mM urea supplementation, and the R6 treatment, which included 19.80 mM of urea-impregnated active nano-zeolite, exhibited the highest NH_3 concentrations. This rise is likely attributed to the buildup of ammonia due to the exhaustion of substrates available for microbial protein synthesis and the lack of absorption processes in the in vitro rumen system (Zhang et al. 2022).

3.2.2. Production of Volatile Fatty Acids (VFA)

Overall, the in vitro VFA concentration for each treatment showed an increase from I2 to I48, with values ranging from 97.57 mM at R4 to 128.77 mM at R3. The VFA concentration findings by Kardaya *et al.* (2009) were similar to those in this study, ranging from 79.5 to 144.5 mM, while Zahera *et al.* (2020) reported higher results of 102.29 mM to 126.69 mM over a 4-hour incubation period. Deitmers *et al.* (2024) observed lower VFA concentrations, between 112.2 and 113.8 mM. The GLM multivariate full factorial analysis indicated no significant interaction between incubation duration and diet treatment. Although diet treatment did not affect VFA concentration, the incubation duration significantly influenced it ($P<0.05$). Duncan Multiple Distance Analysis follow-up tests revealed that the lowest VFA concentrations ($P<0.05$) occurred at incubation periods I2, I4, I8, and I12, while the highest ($P<0.05$) were at I24 and I48. These findings align with Wang *et al.* (2025), who reported that varying mixed ratios of wheat hay with rumen yak and cow increased rumen fluid VFA concentrations at I24, I48, and I72. Saleem *et al.* (2022) also noted an increase in VFA concentrations from I24 to I48. Figure 2 illustrates the average volatile fatty acid production concentration (mM) across different diet treatments during the incubation period.

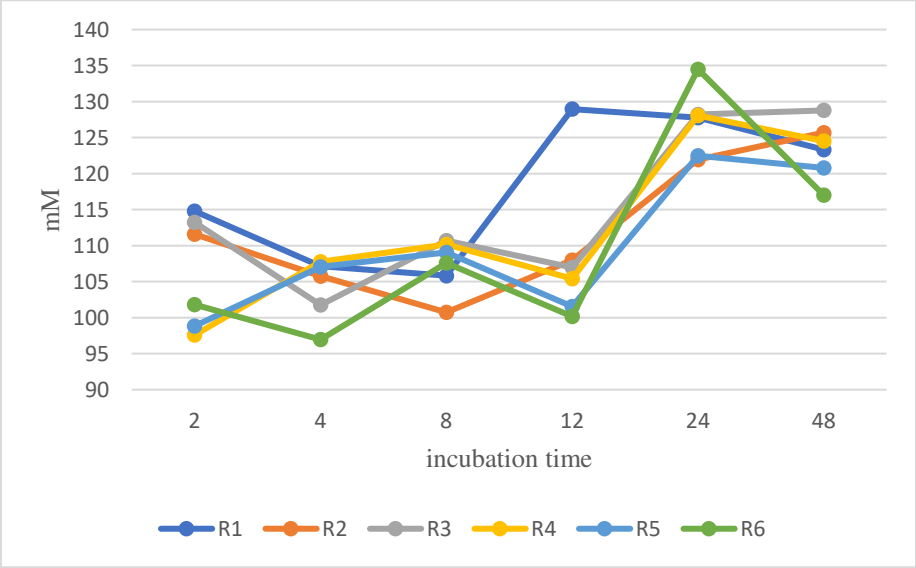

Figure 2 Mean VFA concentration across different dietary treatments throughout the incubation period

The marked variation in VFA concentration observed between the early incubation periods of I2 to I12 and the later stages of I24 and I48 suggests distinct fermentation processes occurring in the rumen in vitro. During the initial I2 incubation, the R1 treatment (control diet) showed the highest VFA concentration at 114.79 mM, closely followed by the R3 treatment (diet with inactive nano-zeolite supplementation) at 113.25 mM, reflecting intense fermentation activity. These VFA levels remain within the normal range of 70-150 mM. The reduction in VFA concentration is attributed to rumen microbes utilizing the fermented products to maintain stable rumen fermentation activity(Zahera *et al.,* 2020). These microbes use the products as sources of energy and nutrients.

The levels of fermented products in I4 to I8 either continued to decline or remained unchanged in most of the treatments. Specifically, the R1 treatment saw a reduction from 107.14 mM to 105.83 mM, and the R2 treatment decreased from 105.78 mM to 100.73 mM. This reduction indicates that microbes persist in using fermented products to build their cellular components. More significant reductions in some treatments might indicate the microbes' proficiency in breaking down the available substrates. According to Syahrir *et al.* (2015), the quantity of microbes in the rumen influences the overall production of VFA. An increase in cellulolytic bacterial cells within the rumen fluid leads to a rise in total VFA production. More substantial decreases in certain treatments could signify the microbes' effectiveness in digesting the available substrates.

During the incubation stages from I8 to I12, there was a rise in VFA concentration. Specifically, in I8, the R3, R4, and R6 treatments showed an increase, while in I12, the R1 treatment reached 128.95 mM before it began to decline, dropping to 127.78 mM in I24 and further to 123.33 mM by I48. This indicates that fermentative activity reached its peak during I8 and I12, followed by a decrease due to the progressively limited availability of substrates.

While there was no statistically significant difference observed between the diet treatments ($P>0.05$), there is an inclination for VFA concentration to vary between diet treatments at each incubation period. The treatment with active nano-zeolite (R4) supplementation recorded the lowest value of 97.57 mM at the 2nd hour, which increased to 105.40 mM by the 12th hour. In contrast, treatments involving urea impregnation (R5 and R6) displayed varied results but generally tended to be higher at certain times. Diets enhanced with urea, nano-zeolite, and combinations of urea-nano-zeolite exhibited variations in VFA concentrations compared to control diets, suggesting that such supplementation could influence rumen fermentation activity. A noticeable difference in rumen VFA values between treatments will become apparent if the feed consumed contains different carbohydrates, particularly crude fiber. Conversely, if the coarse fibers in the complete feed across treatments are nearly the same, the rumen VFA value will not show a significant difference (Doubt *et al.,* 2024). Urea, as a non-protein nitrogen source, can enhance the growth and activity of rumen microorganisms, especially those that use ammonia for microbial protein synthesis (Firsoni and Ansori, 2015). Nano-zeolite has the potential to act as a rumen pH buffer and ammonia adsorbent, which can influence the fermentation environment and VFA production (El-Nile *et al.,* 2021).

3.3.3. Ratio VFA/NH_3

Overall, the VFA/NH_3 ratio for each dietary treatment decreased from incubation I2 to I48, with values ranging from 10.33 for R3 to 5.93 for R6. Kardaya *et al.* (2025) reported VFA/NH_3 ratios between 7.01 and 11.02, which are quite similar to the findings of this study. However, these results differ from those of Fathul and Wajizah (2010), who reported higher ratios of 13.15 to 18.38. The GLM multivariate full factorial analysis indicated no interaction between the duration of incubation (hours) and the diet treatment. While the diet treatment did not affect the VFA/NH_3 ratio, the length of incubation had a significant impact ($P<0.05$) on the VFA/NH_3 ratio in rumen fluid. Further analysis using Duncan's Multiple Range Test

revealed that the lowest VFA/NH3 ratio (P<0.05) occurred at incubation periods I48 and I12, while the highest (P<0.05) was observed at I4 and I8. These findings align with those of Kardaya *et al.* (2009), who found that a rice straw-based diet with added slow-release urea and varying molasses levels increased the VFA/NH_3 ratio in rumen fluid in vitro at incubation I4, then decreased at I8, reaching the lowest values at I24 and I48.

The VFA/NH3 ratio observed in this study remains within the range indicative of effective rumen fermentation, aligning with the findings of Kardaya *et al.* (2025). Despite this, no treatment shows statistically superior efficiency over others. According to Chen *et al.* (2022), the balance between rumen-degradable starch (RDS) and rumen-degradable protein (RDP) influences rumen fermentation characteristics and microbial protein synthesis. Achieving an optimal RDS:RDP ratio can enhance the efficiency of both rumen fermentation and microbial protein production.

3.3.4. pH Level

Overall, the pH level of the fermentation medium for each dietary treatment declines from incubation I2 to I48, with values ranging from 7.14 on R6 to 6.82 on R5. The pH values reported by Kardaya *et al.* (2009)were between 6.81 and 7.32 which are quite similar to the findings of this study Xia *et al.* (2025)reported even higher pH values, specifically between 7.15 and 7.25. The GLM multivariate full factorial analysis indicated no interaction between the duration of incubation (in hours) and the dietary treatment. While the diet treatment did not influence the pH value, the incubation period significantly affected it (P<0.05) in rumen liquids. Further analysis using Duncan's Multiple Range Test revealed that the lowest pH values (P<0.05) occurred at incubation periods I24 and I48, while the highest (P<0.05) were at I2 and I4. These findings align with Saleem *et al.* (2022), who observed that after 48 hours of incubation, the pH of rumen fluid tends to decrease when treating three types of wheat (soft, medium, hard) with three processing methods (milling, dry, and wet rolling).

Research indicates that ruminants consuming grass typically exhibit a physiological ruminal pH between 6 and 7. According to the pH data collected, an increase in incubation time generally leads to a decrease in pH levels, attributed to the production of organic acids by microorganisms within the fermentation medium. . The marked decrease in pH at I48 and I24 compared to the initial hours I2 and I4 indicated that microbial activity in the fermentation medium intensified over time.

In this scenario, it is crucial to acknowledge that while the R1 to R6 treatment did not exhibit a notable change in pH, the differences in incubation duration significantly influenced the fermentation process as a whole. Zahera *et al.* (2022) also observed that pH fluctuations can impact digestibility and fermentability within the rumen system.

4. Conclusion

Diets that include inactive, active, or urea-impregnated nano-zeolites offer a similar level of rumen fermentation efficiency as both control diets and those containing solely urea. Furthermore, the peak feed fermentation efficiency is observed at the 4th and 8th hours of incubation. This indicates that using a combination of nano-zeolite and urea not only sustains optimal rumen fermentation efficiency but also enhances the fermentation process effectively during the early to mid-incubation stages.

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References

Alrez, M., Jammoul, A., Al-Rahmoun, W., & Al-Omar, Y. (2024). The assessment of zeolite addition to diets with or without urea on some productive and physiological indicators in Awassi lambs. *BMC Veterinary Research*, 1-7.

Arshad, M. A., Hassan, F. U., Rehman, M. S., Sharif, M., & Ahmad, N. (2023). Nanotechnology and its role in animal nutrition: A review. *Journal of Animal Physiology and Animal Nutrition, 107*, 1–13. https://doi.org/10.1111/jpn.13708

Camacho, L., Silva, T., Palma, M., Assunção, A., Rodrigues, J., Silva, L., & Detmann, E. (2018). Evaluation of buffer solutions and urea addition for estimating the in vitro digestibility of feeds. *Journal of Animal Science, 97*(2), 922–931.

Chen, P., Li, Y., Shen, Y., Cao, Y., Li, Q., Wang, M., ... Li, J. (2022). Effect of Dietary Rumen-Degradable Starch to Rumen-Degradable Protein Ratio on In Vitro Rumen Fermentation Characteristics and Microbial Protein Synthesis. *Animals*, 2633.

Deitmers, J.-H., Hartinger, T., Gresner, N., & Südekum, K.-H. (2024). Comparison of ruminal fermentation characteristics of two common forages using a coupled in vivo-in situ approach and the in vitro rumen simulation technique RUSITEC. *Animal Feed Science and Technology ,* 1-8.

Detmann, E., Valente, E., Batista, E., & Huhtanen, P. (2014). An evaluation of the performance and efficiency of nitrogen utilization in cattle fed tropical grass pastures with supplementation. *Livestock Science* , 141–153.

Duhan, J. S., Kumar, R., Kumar, N., Kaur, P., Nehra, K., & Duhan, S. (2017). Nanotechnology: The new perspective in precision agriculture. *Biotechnology Reports, 15*, 11–23. https://doi.org/10.1016/j.btre.2017.03.002

El-Nile, A., Elazab, M., El-Zaiat, H., El-Azrak, K.-D., Elkomy, A., Sallam, S., & Soltan, Y. (2021). In Vitro and In Vivo Assessment of Dietary Supplementation of Both Natural or Nano-Zeolite in Goat Diets: Effects on Ruminal Fermentation and Nutrients Digestibility. *Animals*, 1-15.

El-Nile, M. H., Abdel-Latif, M. A., Abd El-Hack, M. E., et al. (2021). Effects of natural and nano-zeolite supplementation on rumen fermentation and nutrient digestibility. *Environmental Science and Pollution Research, 28*, 59878–59887. https://doi.org/10.1007/s11356-021-14706-1

El-Saadony, M. T., et al. (2023). Applications of nanotechnology in the feed industry. *Journal of Nanobiotechnology, 21*, 82. https://doi.org/10.1186/s12951-023-01871-9

Fathul, F., & Wajizah, S. (2010). Addition of Microminerals Mn and Cu in Diet to Sheep Rumen Biofermentation Activity in Vitro. *Journal of Livestock and Veterinary Sciences, 15*(1), 9-15.

General Laboratory Procedures. 1966. Department of Dairy Science. University of Wisconsin, MadisonVogel A.I. 1968. Text Book of Quantitative Inorganic Analysis is including Elementary Instrumental Analysis. Printed in Great Britain by Lowe and Brid one Ltd. London. Pp. 238 – 239

Haque, M. N., Kim, D. H., Lee, H. J., & Lee, S. S. (2019). Synchronization of carbohydrate and nitrogen fermentation in the rumen. *Journal of Animal Science and Technology, 61*, 1–14. https://doi.org/10.5187/jast.2019.61.1.1

Kardaya, D., Wiryawan, K., Parakkasi, A., & Winugroho, H. (2009). Characteristics of Urea Slow Release at Various Molassess Levels in Rice Straw-Based Diets in Vitro. *Journal of Veterinary Animal Science, 13*(3), 177-191.

Kardaya, D., Sudrajat, D., & Wahyuni, D. (2023). Performance of Local Sheep Fed Diets Containing Urea-Impregnated Zeolite. *Journal of Animal Health and Production, 11*(2), 199-205.

Kardaya, D., Sudrajat, D., Wahyuni, D., Gopar, R., & Pramartaa, I. (2025). Rumen Metrics in Sheep Fed Diets Enriched with Urea-Impregnated Nano-Zeolites. *Journal of Animal Health and Production, 13*(2), 223-234.

Khorasani, G. R., & Kennelly, J. J. (2001). Influence of carbohydrate source and buffer on rumen fermentation characteristics, milk yield, and milk composition in late-lactation Holstein cows. *Journal of Dairy Science, 84*(7), 1707-1716.

Kozłowski, K., et al. (2020). Effects of zeolite in ruminant diets. *Applied Sciences, 10*, 5247. https://doi.org/10.3390/app10155247

Menke, K. (1988). Estimation of the energy of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim. Res. Dev, 28*, 7-1.

Menke, K. (1988). Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim Res Dev, 28*, 7-55.

Mousa, K. M., et al. (2022). Nanominerals in ruminant nutrition: Mechanisms and applications. *Small Ruminant Research, 212*, 106692.

Nguyen, T. T., & Dang, P. K. (2018). Effect of zeolite-based slow-release urea on sheep performance. *Asian-Australasian Journal of Animal Sciences, 31*, 1910–1917.

Patra, A. K. (2016). Urea metabolism and nitrogen efficiency in ruminants. *Livestock Science, 185*, 1–10.

Prommachart, R., Phupaboon, S., Matra, M., Totakul, P., & Wanapat, M. (2024). Interaction of a source rich in phytonutrients (fruits peel pellets) and polyunsaturated oil (Tung oil) on in vitro ruminal fermentation, methane production, and nutrient digestibility . *Helion*, 1-11.

Saleem, A., Lira-Casas, R., Gomaa, W., Yang, W., Hucl, P., Randhawa, H., & McAllister, T. (2022). Characterization of various wheat types and processing methods using vitro ruminal batch cultures. *Animal Feed Science and Technology* , 1-12.

Syahrir, S., Mide, M., Islamiyati, R., & Asrianie, A. (2015). Effectiveness of rumen fermentation on mixed feed of rice straw and mulberry biomass with the addition of urea mineral molasses liquid. *Journal of Veterinary Animal Science, 4*(1), 17-22.

Tedeschi, L. O., et al. (2019). Fiber digestion constraints in tropical forages. *Journal of Animal Science, 97*, 327–345.

Van Soest, P. J. (2018). Nutritional Ecology of the Ruminant (3rd ed.). Cornell University Press.

Wang, W., Guo, W., Jiao, J., Ungerfeld, E., Jing, X., Huang, X., . . . Long, R. (2025). Effects of ratios of yak to cattle inocula on methane production and fiber digestion in rumen in vitro cultures. *Journal of Integrative Agriculture, 24*(4), 1270–1284.

Xia, G.-h., Wu, C.-r., Zhou, J.-m., Chen, C., Yang, F.-y., & Hao, J. (2025). Pre-fermented juices for clean and efficient production of mulberry silage: Insights from the

microbiome, metabolome and in vitro rumen fermentation. Environmental Technology and Innovation, 1-13.

Zahera, R., Purwanti, J., & Evvyernie, D. (2022). Ruminal Microbial Population, Fermentability, and Digestibility of Moringa Leaf Supplementation in Dairy Cow Diet in Vitro. *Journal of Nutrition Science and Feed Technology, 20*(3), 117-122.

Zhang, Z., Wei, W., Yang, S., Huang, Z., Li, C., Yu, X., ... & Zhang, X. (2022). Regulation of dietary protein solubility improves ruminal nitrogen metabolism in vitro: role of bacteria–protozoa interactions. *Nutrients, 14*(14), 2972.