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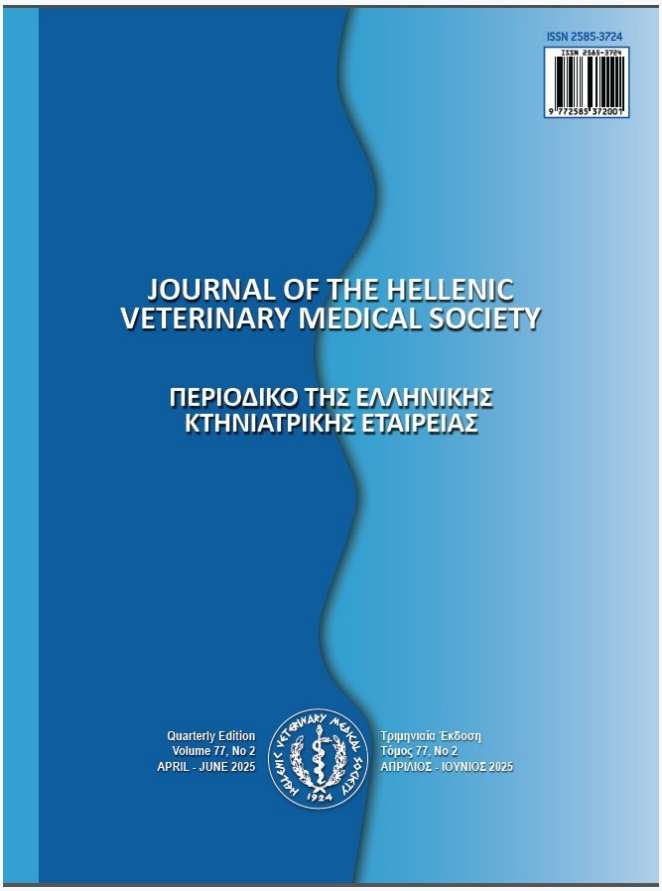

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Effects of rumen protected choline and methionine on in vitro gas production kinetics and the characteristics of rumen fermentation

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ABSTRACT: This study aimed to examine the effects of rumen-protected choline and methionine (RPC and RPM, respectively), with respect to their chemical composition, on in vitro accumulated biogas (BG), kinetics, and in vitro rumen fermentation profile. This study was carried out at different incubation time intervals utilizing an in vitro gas production approach. The experimental design was a 2×2 factorial arrangement in a completely randomized design (CRD) with six replicates per treatment. Total gas production volume and fermentation parameters were measured at 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 hours of incubation. The addition of experimental RPC, RPM, or their mixture did not affect cumulative gas production and gas production kinetics during the various incubation times compared to the control group in terms of b (mL/g DM; P = 0.15), c (fraction per h; P = 0.14), L (fraction per h; P = 0.21), or both (P > 0.05). For all evaluated parameters of the in vitro rumen fermentation profile, including pH, DMD, OMD, and SCFA, no interactions were observed compared to the control (P > 0.05). Overall, the findings showed that the values of the experimental groups for rumen fermentation, gas production kinetics, and in vitro accumulative gas production were identical. Additional studies are required to test the correctness and precision of the current data offered in the paper through using up to update technology.

Keyword: in vitro; VFA; rumen-protected; methionine; choline chloride.

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INTRODUCTION

The ruminants will continue to supply high-quality protein to feed the estimated 10 billion people that will inhabit the planet by the year 2050. However, since ruminants account for approximately 41% of all agricultural greenhouse gas emissions (GHGE) and 17% of all anthropogenic enteric GHGE worldwide, their sustainability has drawn criticism (Pandey et al., 2021). The implementation of the worldwide net-zero emissions program and the critical need to reduce global emissions in 2015 led to an emphasis on GHGE mitigation techniques in ruminant research, particularly through dietary approaches as an essential management instrument (Abbott et al., 2020; Sofyan et al., 2022). For the animal nutrition industry to succeed in the long term and to ensure animal feed security, some promising strategies for mitigating greenhouse gas emissions include inhibiting methanogens, defaunating rumen protozoa, using antibiotics, modifying the ratio of dietary forage to concentrates, and developing new feed sources that are not competing with human food (Zhao & Zhao, 2022). In ruminant production, loss of high biological value proteins and energy due to ruminal fermentation is one of the most significant issues. These could lead to reduced productivity (Ahmed et al., 2016; Valdes et al., 2015) and the release of pollutants into the environment (Calsamiglia et al., 2008).

Though little has been known about how choline ferments in the rumen, preliminary research revealed that ruminal bacteria may quickly and extensively break it down into acetaldehyde and trimethylamine, which can then be further broken down into methane (Neill et al., 1978). According to Pinotti et al. (2002), choline is well known for its functions as a methyl donor, acetylcholine precursor, and precursor of choline-containing metabolites such as phosphatidylcholine, which are critical for lipid metabolism and cell membrane construction.

The top limiting essential amino acid for ruminants is rumen-protected methionine (RPM), especially in those whose milk is sold for volume and component values (Abbasi et al., 2018; Vasconcelos et al., 2006). Supplementing it could improve microbial crude protein (MCP), amino acid (AA) balance, which would decrease blood urea nitrogen and deamination of absorbed AA, both of which are important for dairy animal performance and reproduction (Rhoads et al., 2006). Reducing crude protein (CP) in animal diets has become an increasingly important objective. Rumen-protected limiting amino acids can be supplemented to increase the amount of MP because they increase AA flow in the small intestine, which in turn reduces nitrogen losses, feed costs, and pollution in the environment without negatively affecting animal performance (Guyader et al., 2016; Sinclair et al., 2014).

In particular, as far as we are aware, no published study has examined the rumen fermentation and in vitro production of gas parameters of RPC and RPM at the same time. We therefore postulated that RPM and RPC might generate novel reactions about the aforementioned factors. Thus, the aim of this in vitro experiment was to investigate whether rumen-protected methionine and choline can affect the in vitro gas production and the metabolism of rumen microbes.

MATERIALS AND METHODS

Study preparation

The in vitro rumen experiment was conducted at the Department of Agricultural, Food, Environmental and Animal Sciences, University of Tehran (Alborz, Iran). This investigation was carried out at different incubation time intervals utilizing an in vitro gas production approach. Six replications per treatment were used in the completely randomized design (CRD) with a 2 × 2 factorial structure. The experimental treatments were: (1) Control (CON, n = 6); (2) 60 g/d of RPC [30 g/d of choline ions (CHOL60), n = 6; (3) 30 g/d of RPM [15 g/d of methionine (Met30), n = 6; and (4) 60 g/d of RPC + 30 g/d of RPM [(Met30+ CHOL60), n = 6]. All samples were used for chemical analysis and the in vitro gas test after being dried at 60 °C and ground to fit through a 1-mm sieve (Cyclotech Mill, Tecator, Sweden). According to Feldsine et al. (2002), the samples were examined for dry matter (DM), ash, and crude protein (CP) using the methods of, neutral detergent fiber (NDF), and acid insoluble ash (AIA).

Preparation of RPC and RPM

To separate the three particle sizes (45-63, 63-125, and 125-250 μm), the carrier (bentonite) was first sieved using an auto-stirred sieve (AS 400 control, Retsch, Germany). Liquid choline chloride's pH was brought down to 8, 10, and 12 using NaOH (2 mol/L). Choline chloride temperatures were established at 60, 75, and 90 degrees Celsius. Bentonite was combined with calcium stearate to increase mixability, and then liquid choline chloride (purity 75%; NB Group Ltd., China) was added. For sixty minutes, this solution was agitated using a magnet-

ic stirring device (Ika RCT basic, Germany). The samples were ground (A11, Ika Co., Germany) and sieved using an auto-stirrer sieve after being oven-dried (FP53, Binder Co., Germany) for 24 hours at 75ºC. Subsequently, samples were divided into three core particle sizes (125–500, 500–1000, and 1000–2000 μm). The final weight of the sample formulations was 100 g, and the amount of choline chloride was modified to ensure 100% purity. The same procedures were used for RPM.

Measurement of Biogas production

For in vitro gas operation, rumen fluid was extracted from culled dairy cows at a slaughterhouse and filtered through two layers of cheesecloth into two pre-warmed steel thermoses that had previously been flushed with CO_2. The rumen fluid was transported to the laboratory. Each rumen fluid was filtered through another four layers of cheesecloth into a measuring cylinder. The artificial saliva solution (Kh, 1988) was combined with the ruminal fluid of each animal in a 2:1 (mL/mL) ratio at 39°C while continuously flushed with CO_2. Thirty milliliters of rumen inoculum (RI) mixture were added into each bottle (fermentation unit) under CO_2 flushing. Fermentation units were sealed with rubber stoppers and aluminium caps and incubated at 39°C (96 h) for in vitro gas test. Once all fermentation unit with O_2-free headspace (six bottles for each treatment) were filled, they were immediately closed with rubber stoppers, shaken and placed in an incubator at 39 °C. Gas production was measured at 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 hours of incubation. Using the Pressure Transducer Technique (Theodorou et al., 1994), total GP was measured. The pH value at 48 h of incubation was measured in each fermentation unit (Conductronic pH15, Puebla, Mexico).

Rumen parameters

Following the 96-hour incubation period, measurements were carried out regarding relative gas production (RGP, ml gas 96 h) / (mg/100 mg DMD96) and dry matter degradability (DMD96 mg/100 mg). The short-chain fatty acid (SCFA) concentrations (mmol / 200 mg) were calculated by the equation of (Blümmel et al., 1997). A potentiometer equipped with a glass electrode (pH wireless electrode model HI11102, USA) was used to detect the hydrogen potential (pH) in the liquid.

Chemical analysis and calculation

The TMR samples were ground in a Wiley mill to fit through a 1 mm screen, dried in a forced air oven at 65 °C for 48 hours until they reached a consistent weight, and then placed in plastic bags for further chemical composition analysis and in vitro incubation. The TMR samples were analyzed for nitrogen using the Kjeldahl method (976.05; (Feldsine et al., 2002)), DM (930.15; (Feldsine et al., 2002)), ash content by incineration at 550 °C for 2 hours (942.05; (Feldsine et al., 2002)), and EE by solvent extraction (954.02; (Feldsine et al., 2002)), for the determination of NDF, Alpha-amylase and sodium sulfite were used (Van Soest et al., 1991). The following formulas from Mertens (1997) and Sniffen et al. (1992) were used to determine non-fibrous carbohydrates (NFCs) and total carbohydrates (TCs):

$NFC = 100 - (CP + NDF + EE + Ash)$

$TC = 100 - (CP + EE + Ash)$.

According to the France et al. (2000) model, gas volumes observed (ml/g DM) were fitted using SAS's (2002) NLIN technique to determine the kinetic parameters of GP:

$Y = A \times [1 - e^{-c(t-Lag)}]$

where y is the volume of GP at time t (h); A is the asymptotic GP (ml/g DM); c is the fractional rate of fermentation (/h) and Lag (h) is the discrete lag time prior to any gas being released.

According to (Kh, 1988), the following estimates were calculated for metabolizable energy (ME; MJ/kg DM), in vitro organic matter (OM) digestibility (OMD, g/kg OM), and dry matter (DM) digestibility (DMD, g/kg DM):

ME = 2.20 + 0.136 GP (mL/0.5 g DM) + 0.057 crude protein (CP) (g/kg DM),

OMD = 148.8 + 8.89 GP + 4.5 CP (g/kg DM) + 0.651 ash (g/kg DM),

DMD = 14.88 + 0.889GV + 0.45CP + 0.0651Ash,

where GP is net GP in mL from 200 mg of dry sample after 24 h of incubation.

The ratio of DM degradability in vitro (mg) to the volume (ml) of GP at 24 hours (i.e., in vitro DM disappearance (DMD)/total GP (GP24)) was used to compute the partitioning factor at 24 hours of incubation (PF24; a measure of fermentation efficiency) (Blümmel et al., 1997).

Concentrations of short-chain fatty acids (SCFA; mmol 200 mg^{-1} DM) were computed by Getachew et al. (2002):

$SCFA = (0.0222 \times GP24) - 0.00425$.

Statistical analysis

All statistical analyses were conducted using SAS version 9.4 PROC GLM in a completely randomized design (CRD) with a 2×2 factorial arrangements (SAS Institute Inc., Cary, NC, USA). The following model was used to analyze the data:

$$Y_{ij} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ij}$$

Where: Y = observations; μ = overall mean; A_i = effect of factor A (protein sources, i = 1 to 2); B_j = effect of factor B (level of roughage to concentrate (R:C) ratio, j = 1 to 5), AB_{ij} = interaction between factor A and B, and ε_{ij} = the residual effect. Using Duncan's New Multiple Range Test (DMRT), multiple comparisons between treatment means were conducted (Steel & Torrie, 1960). Statistically significant differences were defined as mean differences with $P<0.05$.

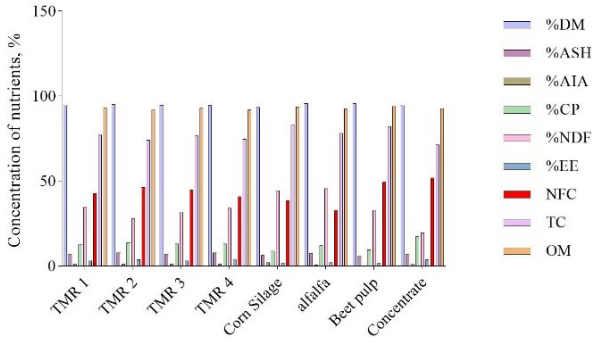

Figure 1. Ingredients and chemical composition of the basal TMR and fermentable substrates of the in vitro experiment (DM%).

RESULTS

Chemical composition

Table 1 and Figure 1 display the chemical composition of the TMR supplement with varying RPM and RPC proportions. Despite a minor variation, the TMRs' chemical composition was similar among treatments. In contrast to the CON group, the inclusion of RPM and RPC raised the percentage of CP, EE, NFC, and TC while decreasing NDF and added in TMR_2 and TMR_4.

In vitro gas production

For each substrate treatment (RPC and RPM), the cumulative in vitro gas production and estimated parameter values derived from the kinetics of gas production models for the substrates under study over 96 hours are shown in Table 2. In contrast to the control group, it is evident that the addition of experimental RPC, RPM, or their mixture had no significant effects ($P > 0.05$) on b (mL/g DM; $P = 0.15$), c (fraction per h; $P = 0.14$), L (fraction per h; $P = 0.21$), or the volume of in vitro asymptotic GP during the various incubation times. A numerical difference was observed among the treatments in the meantime. Accordingly, RPC yielded the least volume of in vitro GP throughout the 96-hour incubation period compared to the CON group ($P = 0.14$).

Rumen fermentation parameters

Table 3 and Figure 2 show the impact of supplementation with RPC and RPM diet on rumen fermentation parameters over 96 hours of incubation. No interactions were found between the controls and any of the investigated parameters of the in vitro rumen fermentation profile, including pH, DMD, OMD, and SCFA ($P > 0.05$).

Table 1. Ingredients and chemical composition of the basal TMR and fermentable substrates of the in vitro experiment (DM%)

Variable[1]	DM	OM	NDF	AIA	CP	EE	CA	TC	NFC
TMR_1 (%)	94.4	92.9	34.6	1.31	12.6	3.15	7.11	77.1	42.5
TMR_2 (%)	95.2	92.2	27.8	1.10	13.9	3.98	7.79	74.3	46.5
TMR_3 (%)	94.6	92.9	31.8	1.05	13.0	3.22	7.10	76.7	44.9
TMR_4 (%)	94.6	92.1	34.1	1.08	13.0	4.24	7.89	74.8	40.8
Corn Silage (%)	93.4	93.7	44.3	2.21	8.9	1.93	6.25	82.9	38.6
Alfalfa (%)	95.6	92.4	45.6	0.67	12.1	2.19	7.56	78.2	32.6
Beet pulp (%)	95.8	93.9	32.9	0.251	9.69	1.98	6.04	82.3	49.3
Concentrate (%)	94.5	92.8	19.7	1.31	17.3	3.94	7.21	71.5	51.8

TMR, total mixed ration; DM, dry matter; OM, organic matter; NDF, neutral detergent fiber; ADF, acid detergent fiber CP, crude protein; EE, ether extract; CA, crude ash; TC, total carbohydrate; NFC, non-fiber carbohydrate

Table 2. In vitro rumen gas kinetics and cumulative gas production after 96 h of incubation (mL/g dry matter (DM))

Treatment[1]	GP parameters			In vitro GP (mL/g DM)										
	b (mL/g DM)	c (h)	L (h)	2h	4h	6h	8h	12h	16h	24h	36h	48h	72h	96h
CON	191	0.06	0.43	24.6	29.8	41.4	69.9	101	119	148	157	177	186	193
RPC	141	0.09	0.72	21.1	24.1	39.4	64.2	85.0	97.9	116	122	132	141	142
RPM	181	0.05	0.15	25.9	28.3	35.6	59.9	93.7	110	133	141	161	177	184
RPC×RPM	168	0.05	-0.21	24.5	27.6	37.1	64.4	93.3	107	126	132	148	168	173
SEM	12.5	0.01	0.25	1.72	2.18	3.58	5.33	6.04	6.72	9.28	10.2	11.5	12.3	12.8
P-value														
RPC	0.01	0.11	0.89	0.17	0.15	0.95	0.91	0.18	0.08	0.04	0.04	0.02	0.04	0.02
RPM	0.51	0.04	0.02	0.18	0.63	0.27	0.37	0.96	0.98	0.77	0.74	0.97	0.48	0.41
RPC×RPM	0.15	0.14	0.21	0.55	0.26	0.64	0.35	0.20	0.18	0.20	0.22	0.18	0.15	0.14

1 Dietary treatments: no supplements (CON); 60 g/d of RPC; 30 g/d of RPM; 60 g/d of RPC + 30 g/d of RPM

1RPC = main effect of ration choline level; RPM = main effect of ration methionine level; RPC × RPM = interaction between ration choline level and methionine level.

Table 3. In vitro rumen fermentation profile after 96 h of incubation (mL/g dry matter (DM))

Treatment[1]	Variable[2]						
	pH	DMD	OMD	ME	NEl	PF24	SCFA
CON	6.41	47.5	39.5	5.84	3.11	1.60	0.65
RPC	6.24	46.6	37.9	5.59	2.92	1.66	0.61
RPM	6.03	43.9	36.5	5.37	2.76	1.65	0.56
RPC×RPM	6.62	44.7	36.2	5.34	2.74	1.69	0.55
SEM	0.26	0.83	0.90	0.14	0.10	0.03	0.02
P-value							
RPC	0.42	0.947	0.312	0.351	0.330	0.174	0.296
RPM	0.99	0.004	0.019	0.020	0.020	0.240	0.022
RPC×RPM	0.16	0.313	0.471	0.466	0.448	0.789	0.419

1 Dietary treatments: no supplements (CON); 60 g/d of RPC; 30 g/d of RPM; 60 g/d of RPC + 30 g/d of RPM

1RPC = main effect of ration choline level; RPM = main effect of ration methionine level; RPC × RPM = interaction between ration choline level and methionine level.

2 DMD, dry matter digestibility; OMD, organic matter digestibility; ME, Metabolizable energy; NEl, Net energy for lactation; PF24, Partitioning factor at 24h; SCFA, Short chain fatty acid

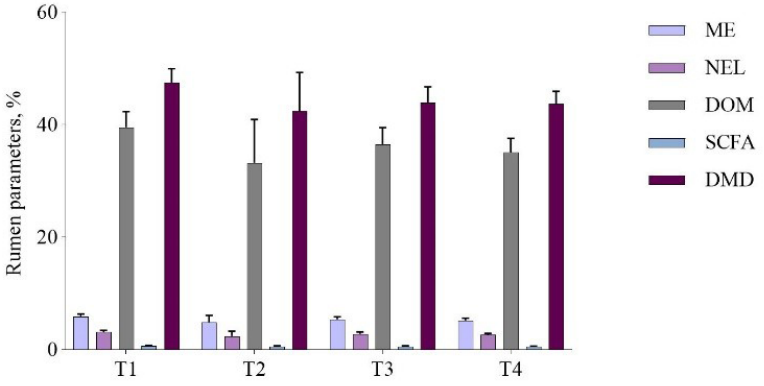

Figure 2. In vitro rumen fermentation profile after 96 h of incubation (mL/g dry matter (DM).

DISCUSSION

Chemical composition

One of the main elements influencing the nutritional value of feeds and ruminal fermentation is chemical composition and nutrient concentration (Kholif et al., 2017). Moderate quantities of fiber enable rumen microorganisms to colonize the ingesta, which may lead to better fermentation rates and improve animal performance, intake, and digestibility (Klopfenstein et al., 2001). Because NFC is a rough estimate of the pool of carbohydrates with different digestibilities than NDF, the range of NFC concentrations in the diets indicated that they might be readily broken down or fermented. Additionally, NFC has been found to positively correlate with the rumen's consumption of ammonia nitrogen (NH_3-N) (Tylutki et al., 2008). The acidic conditions of the forage are responsible for changes in fibrous carbohydrates because they produce acid hydrolysis (Desta et al., 2016), which primarily lowers the NDF content and, to a lesser extent, the ADF content (Contreras-Govea et al., 2011); WSCs and other NFCs increased as a result of these fibers' breakdown (Chen et al., 2019). The crude ash values of the feeds were similar to what Kamalak et al. (2005) and Karabulut et al. (2007) had found. A variety of factors, including climate, soil structure, fertilization, plant species, and type, harvesting time, feed storage conditions, and vegetation period, may be responsible for variations in the nutritional composition of feeds between studies (Canbolat et al., 2013; Kamalak et al., 2005). Since protein is the most expensive nutrient in animal feed, creating natural substitutes for traditional protein meals could be economical. According to Lum et al. (2013), methionine is the leading and second limiting amino acids, respectively, among all the dietary amino acids in ruminant nutrition.

In vitro GP

According to Rodriguez et al. (2015), the GP is typically a reliable indication of fermentability, digestibility, and microbial protein synthesis. Greater gas values demonstrate that the accessibility of rumen microbes to nutrients has been increased (Mahala & Elseed, 2007). In our study, different responses on in vitro GP were expected due to the different RPC and RPM contents of the basal diets used. Moreover, gas production is a reflection of differences in the chemical composition of feedstuffs and has application in predicting their nutritional value. It is also a reflection of the formation of SCFA and the synthesis of microbial biomass (Getachew et al., 1998). Acetate and butyrate production has been associated with higher gas production, whereas propionate production is linked to decreased gas output, according to Getachew et al. (1998). This improvement in GP may be attributed to the low to moderate levels of RPC and RPM, which had beneficial effects on ruminal fermentation, as well as the potential for rumen microbes to break them down and use them as a source of energy (Hart et al., 2008; Wachenheim et al., 1992).

It is widely accepted that approximately 40 percent of the gas generated in the process is generated by fermentation of the feed substrate, with the remaining gas coming from the buffers (Kh, 1988). This is attributed to the fact that the bicarbonate buffer used releases roughly one mole of CO_2 for every mole of VFA produced (Makkar, 2010). On an equal weight basis, the gas generated by the fermentation of casein is only one-third that of the fermentation of carbohydrates, and the gas generation is decreased by 2.48 milliliters per gram of incubated organic matter for every 10 grams of crude protein per kilogram of substrate. The main cause of this decrease in gas production during the fermentation of nitrogenous substances is the ammonia produced, which neutralizes the acids and lowers gas output from the buffer. (Cone & van Gelder, 1999). Raab et al. (1983) examined the role of ammonia in gas production. They suggested using gas production as a way to gauge the protein degradability of typical feeds (such as protein and starchy meals or forages) and based their method on correcting gas readings by determining the amount of ammonia produced.

Rumen fermentation parameters

It is very clear that the response of fermentation patterns depends on harmony between different rumen-protected products in each diet. Short chain fatty acid concentrations are consistent with differences noted in asymptotic gas volume. According to Makkar (2010), changes in gas production have an effect on the production amounts or ratios of VFA. Getachew et al. (1998) reported that in addition to CO_2 and CH_4 produced as a result of fermentation (i.e., direct gas production), CO_2 is also produced upon buffering of SCFA (i.e., indirect gas production) and that molar production of CO_2 equals the molar SCFA production. Since organic acids have hydrophobic properties, they enter the bilayer structure of the bacterial cell's plasma membrane and reduce the growth rate of bacteria by changing the

structure of the membrane and increasing the flowability and permeability (Burt, 2004).

The increase in degradability is reflected in a greater production of SCFA and ME, which is attributed to a greater degradation of carbohydrates [Kholif, 2017], although not evaluated and not observed in the present study. Surprisingly, Moorby et al. (2006) reported linear increases in total VFA and butyrate concentrations and a decrease in acetate with increasing proportion of concentrate in dietary DM, but the concentration of propionate was not affected. Calsamiglia et al. (2008) and Cherdthong et al. (2010) also reported that high concentrate diet fermentation resulted in a greater molar proportion of ruminal propionate. The increase in degradability is reflected in a greater production of SCFA and ME, which is attributed to a greater degradation of carbohydrates, although not evaluated and not observed in the present study (Kholif et al., 2017). Unexpectedly, Moorby et al. (2006) reported linear increases in total VFA and butyrate concentrations and a decrease in acetate with increasing proportion of concentrate in dietary DM, but the concentration of propionate was not affected. Additionally, Calsamiglia et al. (2008) and Cherdthong et al. (2010) also reported that high concentrate diet fermentation resulted in a greater molar proportion of ruminal propionate.

Noftsger et al. (2005) reported that giving dairy cows RPM supplementation enhanced their ability to digest rumen fiber and produce VFA. According to Gajera et al. (2013), animals that were fed a high-forage, maize-based ration that was balanced with RPM either by itself or in combination demonstrated improved CP digestibility. However, the generation of VFA in the rumen was dependent on several factors, including substrates, fiber degradation, bacterial populations, and various reticulorumen sections, in addition to the utilization rate (Bannink et al., 2006). The current investigation shows that over the 96-hour incubation period, the RPM diet produced total VFA at a level comparable to that of the CON group. According to Noftsger et al. (2005), the rumen fermentation index (VFA, NH_3-N) is impacted by the addition of RPM sources. Microbiological activity in the rumen produces VFA, which breaks down cellulose and hemicellulose (Van Zijderveld et al., 2010). Nonetheless, Mulligan et al. (2002) found a correlation between NDF degradability and the formation of total and acetate VFA. In the current investigation, the RPM supplementation groups did not affect total gas production.

Neill et al. (1978) proposed that the rumen is where feedstuff choline is quickly released and thoroughly broken down. According to Atkins et al. (1988), the rumen destroyed 87.5% of the 26 g of choline chloride that is given to the ruminants. According to an experiment, it is estimated that fewer than 2% of choline from each typical feed item will avoid rumen breakdown (Sharma & Erdman, 1989). The researchers found that whereas estimates of rumen-degradable choline for barley, cottonseed meal, fish meal, and soybean meal were equal ($P > 0.05$), fish meal has a significantly higher choline concentration than the other feedstuffs. Therefore, giving cows choline-rich feedstuffs would increase the post-ruminal flow of choline, and therefore might not be suitable for estimating the flow of rumen ingesta using the duodenal flow of choline as a marker (John & Ulyatt, 1979). However, choosing feedstuffs with a high choline content only slightly increases the amount of choline that is post-ruminally accessible, which has minimal bearing on the choline needs of dairy cows (Sharma & Erdman, 1989). Therefore, synthetic sources with significantly greater choline contents and protection against rumen degradation must be created to practically increase the postruminal availability of choline.

CONCLUSION

Overall, this study suggests that the administration of RPC and RPM at 60 g/kg DM and 30 g/kg DM of substrate did not affect cumulative GP and rumen fermentation parameters, or in vitro rumen gas kinetics, throughout the various incubation times (96 h) in comparison to the control group. However, it is known that there are limitations of in vitro techniques evaluating effects of RPC and RPM supplements on the aforementioned parameters. Accordingly, these results necessitate additional studies to provide more information regarding the effects of the supplements on in vitro parameters.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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