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Effect of amino acid supplementation and choline chloride for low protein diet on nitrogen efficiency and methane emission of dairy cows

Efeito da suplementação de aminoácidos e cloreto de colina para dieta pobre em proteínas na eficiência de nitrogênio e emissão de metano de vacas leiteiras

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Highlights

N excretion, retention and CH_4 emission from dairy farms are major nutritional facts.
Dietary N utilization exceled by rumen protected amino acids.
Ruminal and excreta parameters along with CH_4 emission were evaluated.
Environment and animal performance preserved by rumen protected amino acids.

Abstract

Ruminants are one of the largest anthropogenic methane and nitrous oxide emissions. Therefore, the hypothesis was to study the effects of reducing dietary crude protein (CP) level on environmental contaminators when rumen-protected amino acids and choline chloride were supplemented. Sixty Holstein dairy cows were used during the experiment. Test diets were: (1) CD = Control diet with16.2 g of crude protein/ Kg of DM); (2) LM = Low protein diet with 14.2 g of crude protein/ Kg of DM + methionine ; (3) LL = Low protein diet with 14.2 g of crude protein/ Kg of DM + lysine; (4) LML = Low protein diet with 14.2 g of crude protein/ Kg of DM + methionine + lysine; (5) LMLC = Low protein diet with 14.2 g of crude protein/ Kg of DM + methionine + lysine + choline. Dry matter and NDF intake were not different, but the control group received higher CP and ADF compared with other groups ($P < 0.05$). Fecal CP and ADF of control group were lower ($P < 0.05$), but no differences were observed for fecal dry matter (DM) and NDF. Milk yield and protein content were higher for LML and LMLC like control group ($P < 0.05$). Nitrogen intake, urinary N, urinary urea N and total excreta N decreased ($P < 0.05$) when animals fed low protein. There was no difference in ruminal pH and acetate to

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propionate ratio, whereas the ruminal ammonia-N decreased with the low protein (P < 0.05). The 120-h gas production test, showed no difference on the kinetics of digestion and in vitro methane emission. However, the inclusion of DMI in the calculations revealed that low protein can reduce (P < 0.05) methane emission. Overall, our findings indicated that low protein can be compensated for by adding rumen-protected amino acids, not only to maintain the animal performance, but also to decrease nitrogen excretion and methane emission.
Key words: Crude protein. Environmental. Gas production. Methane. Nitrogen retention. Ruminants.

Resumo

Os ruminantes são uma das maiores emissões antropogênicas de metano e óxido nitroso. Portanto, a hipótese foi estudar os efeitos da redução do nível de proteína bruta (PB) na dieta sobre os contaminantes ambientais quando aminoácidos protegidos no rúmen e cloreto de colina foram suplementados. Sessenta vacas leiteiras Holstein foram utilizadas durante o experimento. As dietas teste foram: (1) CD = dieta controle com 16.2 g de proteína bruta / Kg de MS); (2) LM = Dieta pobre em proteínas com 14.2 g de proteína bruta / Kg de DM + metionina; (3) LL = Dieta pobre em proteínas com 14.2 g de proteína bruta / Kg de MS + lisina; (4) LML = Dieta pobre em proteínas com 14.2 g de proteína bruta / Kg de DM + metionina + lisina; (5) LMLC = Dieta pobre em proteínas com 14.2 g de proteína bruta / Kg de DM + metionina + lisina + colina. O consumo de matéria seca e FDN não foi diferente, mas o grupo controle recebeu maior PB e FDA em comparação com os outros grupos (P < 0.05). A PB e FDA fecal do grupo controle foram menores (P < 0.05), mas não foram observadas diferenças para matéria seca (MS) e FDN fecal. A produção de leite e o teor de proteína foram maiores para LML e LMLC como grupo controle (P < 0.05). A ingestão de nitrogênio, N urinário, N urinário urinário e N excreta total diminuíram (P < 0.05) quando os animais foram alimentados com baixa proteína. Não houve diferença no pH ruminal e na relação acetato / propionato, enquanto o N-amônia ruminal diminuiu com o baixo teor de proteína (P < 0.05). O teste de produção de gás de 120 h, não mostrou diferença na cinética de digestão e emissão de metano in vitro. No entanto, a inclusão do CMS nos cálculos revelou que a baixa proteína pode reduzir (P < 0.05) a emissão de metano. No geral, nossos resultados indicaram que o baixo teor de proteína pode ser compensado pela adição de aminoácidos protegidos no rúmen, não apenas para manter o desempenho animal, mas também para diminuir a excreção de nitrogênio e a emissão de metano.
Palavras-chave: Proteína bruta. Ambiental. Produção de gás. Metano. Retenção de nitrogênio. Ruminantes.

Introduction

Ruminants produce meat and milk, by converting human-indigestible plant into human edible food products via adaptation of the ruminal digestive tract microbiome (Van Zanten, Meerburg, Bikker, Herrero, & De Boer, 2016). On the other hand, ruminants are one of the pollutant sources (Greening et al., 2019). Nowadays the studies are concentrating on decreasing of level of protein in dairy cow diets, because low protein in the diets can set low excreta of nitrogen (N). One of the most useful strategies is to reduce dietary crude protein (CP) level to reduce N excretion through urine and manure (Lee et al., 2012b). However, reducing CP level can decrease metabolizable protein supply to less than minimum requirements (National Research Council [NRC], 2001), which can reduce

animal efficiency (Lee et al., 2011). Yet adding individual amino acids (aa) to metabolizable protein supply may compensate cow productivity. Methionine and lysine have been suggested as the first-limiting amino acids on corn-based diets (NRC, 2001). Thus, adding rumen-protected (RP) DL-methionine (RPMet) and choline (RPChol) as methyl donors and rumen-protected L-lysine (RPLys) may improve dairy cow efficiency along with decreased level of N excreta to the environment (Swanepoel, Robinson, & Erasmus, 2010; Giallongo et al., 2016; Zenobi et al., 2018). However, some studies implied protein sources containing of rumen undegradable protein (RUP) are improving dairy cow performance (Amanlou, Farahani, & Farsuni, 2017; Rehman et al., 2020), but in most studies applying of essential amino acids supplementing showed high efficiency in nitrogen retention (Noftsger, St-Pierre, & Sylvester, 2005; Gomez et al., 2011; Lee et al., 2012a; Lee et al., 2012b). Beside N excretion, methane production by ruminal methanogens seems important.

Dominant methanogens, *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium,* being the most dominant rumen methanogens (about 74% of the biomass), utilize molecular hydrogen (H_2) for reducing carbon dioxide (CO_2) to methane (CH_4) (Leahy et al., 2010; Thauer, 2012; Henderson et al., 2015). At the presence of H_2, as the basal substrate for ruminal methanogenesis (produced through multiple carbohydrate fermentation pathways) together with the volatile fatty acids (VFA) and CO_2 (Seshadri et al., 2018; Solden et al., 2018; Stewart et al., 2018), ruminal methanogens can use acetate, formate, ethanol and methyl compounds as substrates (Kelly et al., 2014; Henderson et al., 2015; Lambie et al., 2015; Li et al., 2016). Along with environmental concerns, it is believed that produced methane in the rumen cause an energy loss of about 2 to 12% in ruminants metabolizable energy (ME) which could consequently reduce total efficiency of the animal performance (Johnson & Johnson, 1995; Gill, Smith, & Wilkinson, 2010; Hynes, Stergiadis, Gordon, & Yan, 2016; Serrano, Cruz, Coneglian, & Branco, 2020). Since ruminants contribute in greenhouse gas emission, variety of programs are underway to decrease the methane production (Martin, Morgavi, & Doreau, 2010; Buddle et al., 2011; Kiggundu, Nantongo, Kayondo, & Mugerwa, 2019). To-date, direct inhibition of the methanogen bacteria, whether by vaccine and or anti-microbial compounds, was assumed to be the main strategy yet (Wedlock, Janssen, Leahy, Shu, & Buddle, 2013; Hristov et al., 2015; Weimar et al., 2017; Henderson, Cook, & Ronimus, 2018) feed or nutritional management could be an alternative policy (Kiggundu et al., 2019). Nutritional management approaches can be used to regulate the supply of methanogen bacteria substrates, like molecular hydrogen and ammonia (Buddle et al., 2011; Wu et al., 2018; Sun, Aguerre, & Wattiaux, 2019; van Lingen, Jonker, Kebreab, & Pacheco, 2021).

Ruminal methanobacteria use ammonia to propagate and simultaneously produce microbial protein for the host animal (Jarrell & Kalmokoff, 1988; NRC, 2001). It is of note that the main source of the ammonia is the rupture of rumen-degradable protein (RDP) (NRC, 2001; Kalscheur, Vi, Glenn, & Kohn, 2006). To date many approaches have been set by scientists to address greenhouse gas emission reduction strategies of which livestock diet RDP restriction seems to be more practical. Restricting RDP level can influence methane production as well as nitrate excretion (NO_3^-) (Sun et al., 2019).

Thus, the objective of current study was to investigate the effects of low protein diets supplemented with rumen-protected Met, Lys and choline on N utilization, N excretion and methane emission in Holstein lactating dairy cows.

Material and Methods

Animals, treatments and experimental design

A total number of sixty Holstein dairy cows (30 primiparous and 30 multiparous) were used in a two-phase experiment (each phase was 30 cows; 15 primiparous and 15 multiparous), all cows were treated according to guide to the care and use of experimental animals (I.C.o.A.C. 1995) Cows had body weight of 650±35 Kg and body condition score≅ 3, cows were housed individually in free-stall barns. Every phase was 28 days, first 21 days were for adaptation period and 7 last days considered for sampling. The experiment was conducted in a completely randomized block design, animals of each phase were randomly assigned to one of the five experimental diets, based on their days in milk (DIM), milk yield and parity. Experimental diets, shown in Table 1, were (1) CD = Control diet with16.2 g of crude protein/ Kg of DM); (2) LM = Low protein diet with 14.2 g of crude protein/ Kg of DM + methionine ; (3) LL = Low protein diet with 14.2 g of crude protein/ Kg of DM + lysine; (4) LML = Low protein diet with 14.2 g of crude protein/ Kg of DM + methionine + lysine; (5) LMLC = Low protein diet with 14.2 g of crude protein/ Kg of DM + methionine + lysine + choline. The diets were formulated to meet the nutrient requirements of cows producing 45 kg/d of milk, 3.3% milk fat and 2.9% milk true protein except MP requirements (NRC, 2001). The experimental diets were provided as total mixed ration at 07h30, 15h30 and 23h30, ad libitum for 5 to 10% of refusals. The RPMet, RPLys and RPChol were fed top-dressed immediately after the diets were delivered. The cows were milked daily at 06h30, 14h30 and 22h30

Table 1
Ingredient and chemical composition of the experimental diets fed in the trial (% of DM)

Item	Experimental diets[1]				
	Control	LM	LL	LML	LMLC
Ingredients, % of DM					
Corn silage	20.56	20.41	20.41	20.41	20.41
Alfalfa there	21.49	21.33	21.33	21.33	21.33
Wheat straw	4.08	4.05	4.05	4.05	4.05
Cottonseed, whole	5.06	5.02	5.02	5.02	5.02
Beet sugar pulp	3.73	3.71	3.71	3.71	3.71
Molasses	1.32	1.31	1.31	1.31	1.31
Barley grain, ground	12.79	14.33	14.33	14.33	14.33
Corn grain, ground	12.15	13.96	13.96	13.96	13.96
Corn gluten meal	5.03	3.55	3.55	3.55	3.55
Soybean meal (solvent extracted)	8.23	4.49	4.49	4.49	4.49

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continuation...

Soybean seeds, whole, heated	0.82	0.82	0.82	0.82	0.82
Corn germ	0.31	0.50	0.50	0.50	0.50
Wheat bran	0.97	2.57	2.57	2.57	2.57
Calcium soap of fatty acids	0.67	1.22	1.22	1.22	1.22
Salt	0.17	0.17	0.17	0.17	0.17
Urea	0.29	0.29	0.29	0.29	0.29
Calcium phosphate (Di)	0.31	0.31	0.31	0.31	0.31
Calcium carbonate	0.41	0.41	0.41	0.41	0.41
Magnesium oxide	0.17	0.17	0.17	0.17	0.17
Sodium bicarbonate	0.86	0.85	0.85	0.85	0.85
Mineral premix[2]	0.28	0.27	0.27	0.27	0.27
Vitamin premix[3]	0.29	0.27	0.27	0.27	0.27
Rumen-protected Methionine (RPMet) g/d	-	30	-	30	30
Rumen-protected Lysine (RPLys) g/d	-	-	130	130	130
Rumen-protected Choline (RPChol) g/d	-	-	-	-	60
Composition, % of DM					
CP	16.2	14.2	14.2	14.2	14.2
RDP	10	9.1	9.1	9.1	9.1
RUP	6.2	5.1	5.1	5.1	5.1
NDF	32.9	32.7	32.7	32.7	32.7
ADF	24.3	24.0	24.0	24.0	24.0
NFC	41.6	43.3	43.3	43.3	43.3
Ca	0.8	0.9	0.9	0.9	0.9
P	0.4	0.4	0.4	0.4	0.4
NEL, Mcal/kg	1.52	1.52	1.52	1.52	1.52
NEL balance, Mcal/d	1.9	2.2	2.2	2.2	2.2
Protein supply, g/d					
RDP supply	2913	2667	2667	2667	2667
RDP balance	116	-166	-166	-166	-166
RUP supply	1815	1507	1507	1507	1507
RUP balance	172	-264	-264	-264	-264
MP supply	3236	2878	2878	2878	2878
MP requirements	3087	3104	3104	3104	3104
MP balance	149	-226	-226	-226	-226
Lys/Met	3.04	3.06	3.06	3.06	3.06

[1]Control = diet containing 16.2% CP; LM = diet containing 14.2% CP supplemented with RPMet Mepron [Evonik Nutrition & Care GmbH, Hanau, Germany]; LL = diet containing 14.2% CP supplemented with RPLys [Timet; VETAGRO S.p.A.; Reggio Emilia, Italy]; LML= diet containing 14.2% CP supplemented with RPLys and RPMet; LMLC= diet containing 14.2% CP supplemented with RPLys and RPMet and RPChol ReaShure (Balchem Corp., New Hampton, NY).
[2]The premix contained (%, as-is basis): trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; 0.48; limestone, 37.2; selenium premix, 0.07; and 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg;
[3]Vitamin ADE premix, vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.

Sampling, chemical composition and analyses

Diets and ort were recorded three times a week during the experiment. Individual feed ingredients, TMR and ort were sampled three times a week and composited by week and treatment on equal weight and frozen at −20°C until analyses. Samples were air dried and ground in a Wiley mill. (A. H. Thomas Co., Philadelphia, PA) adjusted to 1-mm sieve, and analyzed for DM (AOAC, 2005); method 930.15), CP (method 984.13), NDF (neutral detergent fiber) and ADF (acid detergent fiber) were determined according to Van Soest, Robertson and Lewis. (1991) using a heat-stable α-amylase Sigma (Number A3306, Sigma Chemical Co., St. Louis, MO).

Milk and milk composition

Milk yield was recorded and sampled in three consecutive milking during three days of last experimental week. Samples were composed proportionally according to the production of each milking and added to a 100 ml falcon tube. The milk samples were analyzed for milk composition using Lactoscan (Milk Analyzer, For Laboratory Use).

Urine and fecal sampling

Urine samples (about 300 ml per cow) were taken by massaging the vulva. A portable pH meter was used to measure the pH immediately after sampling, then acidified to pH < 3.0 with 2 M H_2SO_4 pre storage at −20°C samples were diluted with distilled water (1:10) for subsequent analyses (Lee et al., 2012b). Samples of acidified urine were composited according to the cow and treatment, then analyzed for urea N (enzymatic Urease-GLDH method, kit no. 1400030), uric acid (enzymatic colorimetric TOOS method, kit no. 140031) and creatinine (enzymatic Jaffe method, kit no. 1400009) using commercial kits (Pars Azmoon Co., Tehran, Iran) according to the instructions of the manufacture. Daily urine volume was estimated using body weight (BW) and urinary creatinine concentration which was calculated by the equation of 29 (mg/kg) × BW (kg) × [1/urinary creatinine (mg/L)] (Valadares, Broderick, Valadares, & Clayton, 1999). Fecal samples (approximately 500 g of each sampling) were collected three times a day (every 8 hours) in three consecutive days of last week. Samples were oven-dried (48 h at 65°C) and then composited by cow and day on equal dry weight basis. The composite samples analyzed for CP, NDF and ADF (Amanlou et al., 2017).

Ruminal parameters and fermentation

Ruminal fluid samples were collected about 3-4 h after the morning feeding on the 21st of each period, using a rumen sampler (Geishauser, Linhart, Neidl, & Reimann, 2012). Ruminal fluid samples were strained and sub-samples of approximately 15 ml were performed. One of the sub-samples was acidified with 3 ml of 50% H_2SO_4 for NH_3 analysis (Broderick & Kang, 1980) and another sub-sample was diluted with 3 ml of 25% metaphosphoric acid for VFA analysis (Larsen, Hansen, Weisbjerg, & Lund, 2020) and frozen at −20°C until onset analyses.

Gas production technique

Samples of every treatment were ground in a Wiley Mill having 2 mm screening (Arthur H. Thomas, Philadelphia, PA, USA), and then 300 mg were weighed into glass vials (50 ml volume). Synthetic saliva was prepared according to McDougall (1948). Ruminal contents of at least three slaughtered dairy cattle obtained from slaughter house were strained through 3-layer cheese cloth to the pre-warmed flasks leaving no headspace, rumen liquor flasks were taken to the laboratory (Lutakome et al., 2017). To form a uniform aliquot, all liquors were blended in 39ºC under the flush of CO_2 and mixed with the artificial saliva (1:2 v/v) and loaded (20 ml) to the glass vials. Each experimental diet sample was incubated in five replicates. Five vials of only digestion medium were used as blank vials. The vials were immediately sealed and placed in a shaker platform adjusted to 39ºC. Data for gas production of each vial were recorded after 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h of incubation using of water replacement assay (Fedorah & Hrudey, 1983; Shirmohammadi et al., 2020). Gas production kinetic was described as the following model: $y=A(1-e^{(-ct-lag)})$, where Y is the volume (mL) of produced gas at time t; A is the gas production from the soluble and insoluble fraction (mL); c is the constant rate of gas production (ml h^{-1}); t is the incubation time (h) and lag is the lag time (h).

Ex-vivo methane emission

Along with gas production test, about 200 mg of ground (2mm) samples of each treatment were weighed into three graduated syringes (Fortune glass gas syringes (100 ml), Fortuna Co., Germany) and prepared digestion medium were loaded (20 ml) to each syring and three syrings filled just with digestion medium as blank and placed in a shaker platform adjusted to 39ºC. After 24 h of incubation the cumulative gas volumes of each sample were documented and about 5 ml of upper gas phase of each syring were sampled and injected into a gas chromatograph (GC-Clarus 500-PerkinElmer) equipped with the column (Agilent Technologies, HP-5ms GC Column, 30 m, 0.25 mm, 0.25 µm, 7 inch cage (CATALOG NO. 19091S-433)). The procedure run time was 65 min,samples were injected manually, initial temperature was 40 (held for 10 min), then the prosedure of 10 deg per min was continued up to 290 deg which was held for 30 min. The carrier gas was Helium (Adejoro, Hassen, Akanmu, & Morgavi, 2020), then quantitative methane measurement were calculated by external standard.

Statistical analysis

Collected data for DMI and milk yield and composition were analyzed using PROC MIXED of SAS (SAS software version 9.4) as repeated measurement with the following model:

$$Y_{ijk} = \mu + C_i + T_j + CT_{ij} + P_k + TP_{jk} + e_{ijk}$$

where Y_{ijk} is the dependent variable, µ is the overall mean, C_i is the cow, T_j is the jth treatment (experimental diet), CT_{ij} is the cow × treatment (experimental diet) interaction , P_k is the experimental phase (phase 1 and 2), and TP_{jk} is the treatment × phase interaction, with the error term eijk assumed to be normally distributed.

Collected data for nutrient intakes, fecal parameters, urinary and fecal N losses and ruminal parameters were analyzed using

PROC GLM of SAS (SAS software version 9.4) as the following model:

$$Y_{ij} = \mu + C_i + T_j + P_k + PT_{jk} + e_{ij}$$

where Y_{ijk} is the dependent variable, μ is the overall mean, Ci is the cow, T_j is the jth treatment (experimental diet), P_k is the experimental phase (phase 1 and 2), and TP_{jk} is the treatment × phase interaction, with the error term e_{ijk} assumed to be normally distributed. P-value of 5% (P≤ 0.05) were declared as significant difference among treatments.

Results and discussion

Dry matter intake was higher for control (P<0,01) than those obtained for the others (Table 2). As predicted, higher amount of CP was consumed by control group compared to the other groups (P<0. 01). We found no differences for the NDF and ADF intake among the test groups. Fecal DM and CP were not different among the test diets (Table 2), but the other fecal parameters (NDF and ADF) were differences (P<0.05).

Table 2
Effect of crude protein level and rumen-protected aminoacids supplementation on nutrients intake and fecal parameters in dairy cows

Item[2]	Experimental diets[1]					SEM[3]	P-Value
	Control	LM	LL	LML	LMLC		
Nutrient intake, kg/d							
DM	27.29	26.53	26.23	26.84	26.80	0.398	<.01
CP	4.42[a]	3.77[b]	3.72[b]	3.81[b]	3.80[b]	0.057	<.01
NDF	8.98	8.68	8.58	8.78	8.76	0.130	0.29
ADF	6.63[a]	6.37[ab]	6.29[b]	6.44[ab]	6.43[ab]	0.096	0.16
Fecal parameters %							
DM	21.52	21.13	21.68	20.87	21.35	0.315	0.41
CP	38.22[b]	40.33[a]	40.17[a]	39.90[a]	40.19[a]	0.493	0.09
NDF	47.22	48.85	48.83	48.55	48.27	0.544	0.02
ADF	33.18[b]	34.65[a]	34.75[a]	34.46[a]	34.75[a]	0.382	0.02

Within a row, means without a common superscript letter differ (P<0.05).
[1]Control = diet containing 16.2% CP; LM = diet containing 14.2% CP supplemented with RPMet Mepron [Evonik Nutrition & Care GmbH, Hanau, Germany]; LL = diet containing 14.2% CP supplemented with RPLys [Timet; VETAGRO S.p.A.; Reggio Emilia, Italy]; LML= diet containing 14.2% CP supplemented with RPLys and RPMet; LMLC= diet containing 14.2% CP supplemented with RPLys and RPMet and RPChol ReaShure (Balchem Corp., New Hampton, NY).
[2]DM= Dry matter; CP= Crude protein; NDF= Nutral detergent fiber; ADF= Acid detergent fiber
[3]SEM: Standard errors of mean.

Interestingly numerically reduced DMI, (average of 690 gr), an indicator of reduced CP level, differed from previous studies (Lee et al., 2011; Huhtanen & Hetta, 2012). A depression of 1.5 kg/d in DMI were reported by Lee et al. (2012a). Variation in the amount of reduced DMI was due to the reduced CP level percentage. in our study we decreased CP from 16.2 to 14.2 (-12.34%) while Lee et al. (2012a) decreased CP from 15.7 to 13.6 (-13.37%). Therefore both of the percentage of the reduced level and concentration of CP are important. In contrary, Amanlou et al. (2017) increased CP level from 16 to 19 and 21 at first 21 days after calving, they reported that DMI were increased by increasing CP concentration to 19 but no significant differences was shown between 19 and 21 levels, resulting that optimum CP level influenced DMI must be considered. Ruminal fibrolytic bacteria is influenced by RDP (Russell, O'connor, Fox, Van Soest, & Sniffen, 1992). Reduced RDP that compensated with rumen protected AA, may decrease digestibility of the fiber in DMP diets. In our study fecal parameters such as NDF and ADF shows that fiber digestion has decreased which is in consistent with former studies (Broderick, Stevenson, Patton, Lobos, & Colmenero, 2008; Lee et al., 2012a,b). However Noftsger et al. (2005) reported an increase in NDF and organic matter digestibilities when RPAA was used In the diet, the main differences could be the CP level where they designed to have 18.4 CP in the experimental diet versus 14.2 CP of this study and the source of RPAA of this study and their study (Mepron vs. HMB and HMBi).

Daily intake of N for the control was higher than those obtained for the other treatments (P<0.01). Milk yield was higher for control, LML and LMLC (P<0.), whereas no difference was found in milk protein concentration being 3.06% on average. Calculated milk N for control, LML and LMLC was higher than the LL (P<0.05). Urinary N excretion decreased significantly from 169.21 g/d of control to 123.71 g/d of LL (P<0.05). It should be noted that there were no differences among the other low protein diets, however there was an interaction effect of the experimental phase and treatment in the case of urine-N (P<0.004). A significant decrease for urinary urea-N g/d was observed (P=….0.05) when rumen protected products were used. Urinary urea-N ratio of total urinary N (Table 3) was significantly reduced from 54.21% of control to 36.47% and 36.83% for LL and LM, respectively (P<0.05). The lowest amount of fecal N was for AMP while the highest was for LPDM (P<0. 01), whereas total excreta N loss was achieved for control diet (P<0. 01).

Table 3
Effect of CP level and rumen-protected aminoacids supplementation on milk production and N balance in dairy cows

Item	Experimental diets[1]					SEM[2]	P-Value
	Control	LM	LL	LML	LMLC		
N intake, g/d	707.34^{a}	602.84^{b}	595.99^{b}	609.72^{b}	608.91^{b}	9.126	<.01
Milk yield, kg/d	42.90^{a}	41.83^{b}	41.39^{b}	42.32^{ab}	42.34^{ab}	0.376	0.07
Milk protein, %	3.09	3.03	3.02	3.07	3.08	0.024	0.23
N secretion and excretion, g/d							
Milk N[3]	207.48^{a}	198.91^{bc}	195.88^{c}	203.92^{ab}	204.43^{ab}	2.454	0.01
Urine N[4]	169.21^{a}	128.43^{b}	124.80^{b}	123.71^{b}	124.95^{b}	1.877	<.01
Urinary urea-N (UUN)	91.70^{a}	47.30^{b}	45.53^{b}	47.75^{b}	46.87^{b}	1.032	<.01
UUN ÷ total urinary N, %	54.21^{a}	36.83^{c}	36.47^{c}	38.59^{b}	37.50^{bc}	0.430	<.01
Fecal N	238.84^{b}	252.03^{a}	251.06^{a}	249.38^{a}	251.81^{a}	3.079	0.02
Total excreta N	408.06^{a}	380.46^{b}	375.86^{b}	363.72^{b}	373.09^{b}	4.645	<.01
As proportion of N intake, %							
Urine N	23.91^{a}	21.31^{b}	20.95^{c}	20.30^{e}	20.52^{d}	0.064	<.01
Fecal N	33.79^{b}	41.84^{a}	42.14^{a}	41.00^{a}	41.27^{a}	0.396	<.01
Total excreta N	57.70^{c}	63.15^{a}	63.09^{a}	61.30^{b}	61.79^{b}	0.417	0.01

Within a row, means without a common superscript letter differ (P<0.05).
[1]Control = diet containing 16.2% CP; LM = diet containing 14.2% CP supplemented with RPMet Mepron [Evonik Nutrition & Care GmbH, Hanau, Germany]; LL = diet containing 14.2% CP supplemented with RPLys [Timet; VETAGRO S.p.A.; Reggio Emilia, Italy]; LML= diet containing 14.2% CP supplemented with RPLys and RPMet; LMLC= diet containing 14.2% CP supplemented with RPLys and RPMet and RPChol ReaShure (Balchem Corp., New Hampton, NY).
[2]SEM: Standard errors of mean.

Adding both RPMet and RPLys and also RPChol resulted to the equal amount of milk production and milk N secretion, and also we found no differences in milk protein adding either one or both of RPAA to diets, which were consistent with that reported by Lee et al. (2012b). Similar to our results Giallongo et al. (2016) reported a reduction in milk yield when just RPMet or RPLys was used in diet as additive. Contrary to our findings Jenkins et al. (2020) reported that there is no significant difference between control and RPAA addition. A meta-analysis conducted by Huhtanen and Hristov (2009) reported that dietary N intake is the primary source for determining N losses in dairy cattels. The amount of N not being retained in the animal body tissues or in its milk will be excreted in feces and urine, excreted N not only can contribute in water pollution and nitrogen oxides (NO_x) emission, but also will participate in microparticle formation of atmosphere (Hristov et al., 2019). Agriculture anthropogenic nitrous oxide (N_2O), one of the major greenhouse gasees, emission is about 60% in which much of N_2O is from livestock excreta that transformed as N deposit to soil (Hou, Velthof, & Oenema, 2015; Hamamoto, Uchida, von Rein, & Mukumbuta, 2020). As predicted the cows that received low protein showed a significantly less N due to lower

dietary CP level. Some studies indicated a reduction in urinary N and urinary urea N (UUN) excretion when the animals received low protein diets (Lee et al., 2012a,b) which is in agreement with ours where the urine N and UUN have decreased on average of 25.8% and 48.9%, respectively. More fecal N proportion of DMP diets were reported in previous studies (Lee et al., 2012a,b) being inline with the results of current study, but the most important source of ammonia emission from dairy cattle manure is the UUN, beside that the urinary N is responsible for about 87% of ammonia N effusion (Lee et al., 2011), Dijkstra, Bannink, Bosma, Lantinga, & Reijs (2018) reported that there is a vast variation in excreted urinary N in comparison to fecal N, offering opportunity for dietary manipulation to decrease urinary nitrogen excretion. resulting reducing of the N excreted from urine may be an effectual method to protect the environment.

No differences were observed in ruminal pH (Table 4), but was found an interaction between experimental phase and treatment in the case of ruminal pH (P=0. 04). As predicted, the ammonia-N fell significantlly (P<0. 01) from 12.52 mg/dL in control diets to 11.52 mg/dL in LL diet. The highest total VFA, acetate and propionate were observed with DML, whereas nodifferences were detected for the other VFAand the acetate to propionate ratio.

Ruminal fermentation parameters are shown in Table 4. In this study we found no significant differences in ruminal pH, however Jenkins et al. (2020) reported a decrease in ruminal pH when dietary MP was high and also HMBTA (2-hydroxy-4-methylthio-butanoic acid) were used as supplementary which being due to dietary constitutes differences. In line with our results Noftsger et al. (2005) reported no changes in ruminal pH in the diets that contained different levels of CP. Peculiarly in the present study ruminal pH were above 6 while both of the aforementioned studies have reported pH of less than 6. We, in this study, assumed that the amount of corn silage as percent of DM used in the diets could be the origin of this difference. we used about 20% of DM, whereas they both used around 30% of DM.

Table 4
Effect of CP level and rumen-protected aminoacids supplementation on ruminal parameters in dairy cows

Item	Experimental diets[1]					SEM[2]	P-Value
	Control	LM	LL	LML	LMLC		
Ruminal Parameters							
pH[3]	6.33	6.37	6.45	6.48	6.39	0.076	0.633
Ammonia-N (mg/dL)	12.52^{a}	11.56^{b}	11.52^{b}	11.63^{b}	11.59^{b}	0.099	<.0001
Total VFA (mmol/L)	86.51^{b}	89.20^{ab}	87.91^{ab}	91.56^{a}	90.02^{ab}	1.195	0.045
Acetate, mol/100 mol	51.24^{b}	52.72^{ab}	51.65^{b}	54.02^{a}	53.07^{ab}	0.709	0.056
Propionate, mol/100 mol	22.67^{b}	23.72^{ab}	23.53^{ab}	24.38^{a}	23.97^{ab}	0.378	0.035
Butyrate, mol/100 mol	9.98	10.18	10.04	10.47	10.30	0.159	0.212
Valerate, mol/100 mol	1.46	1.41	1.50	1.48	1.49	0.038	0.498
Isovalerate, mol/100 mol	1.16	1.19	1.19	1.21	1.19	0.039	0.881
Acetate: propionate	2.26	2.23	2.20	2.22	2.22	0.025	0.514

Within a row, means without a common superscript letter differ (P<0.05).
[1]Control = diet containing 16.2% CP; LM = diet containing 14.2% CP supplemented with RPMet Mepron [Evonik Nutrition & Care GmbH, Hanau, Germany]; LL = diet containing 14.2% CP supplemented with RPLys [Timet; VETAGRO S.p.A.; Reggio Emilia, Italy]; LML= diet containing 14.2% CP supplemented with RPLys and RPMet; LMLC= diet containing 14.2% CP supplemented with RPLys and RPMet and RPChol ReaShure (Balchem Corp., New Hampton, NY).
[2]SEM: Standard errors of mean.
[3]experimental phase × treatment interaction (P = 0.039).

Ammonia-N was greater for AMP than the other experimental treatments, Sun et al. (2019) reported that decreasing RDP level can be an effective way to reduce NH3-N, on the other hand Jenkins et al. (2020) reported that adding HMTBA in both low and high MP had tendency to increase the NH3-N pool yet they didn't conclude on the mode of action yet. it was suggested that addition of HMTBA may have stimulated the ruminal bacteria to degrade more dietry protein. Rechecking the ammonia-N of mentioned studies reveals that the amount of RDP can have an important role in ammonia-N pool, anyway as mentioned by an old study minimum of 5 mg/dl of ammonia-N can produce maximum ruminal bacteria (Satter & Slyter, 1974).

Total VFA, acetate and propionate was greater for LPD groups than control group which is inline with those reported by Jenkins et al. (2020), however they found a reduction in acetate, meanwhile Noftsger et al. (2005) reported non significant differences in ruminal total VFA, acetate and propionate when HMB, HMBi or DL-Met were used. Deep analysing the tables may reveal that amount of intake NDF and NFC could be suitable variables to explain the VFA component differences, in our study the amount of intake NDF was about 8.8 and Jenkins et al. (2020) was about 8.3, but for Noftsger et al. (2005) it was 5.6. On the other hand we found no differences in acetate: propionate ratio like that reported by Noftsger et al. (2005), while Jenkins et al. (2020) reported a decrease in the later parameter, which can

be as a result of NFC fermentation, where our diets NFC was 43 which is similar to that of Noftsger et al. (2005).

Although did significant, the initial incubation times of gas production were numerically higher for LMLC (Table 5), but from 8 h of incubation the gas production changed up to the last times of incubation (120 h) where the control diet had the numerically highest value (334.40 ml/g of DM) and the LL diet showed the lowest concentration (327.84 ml/gDM). Total gas production at 24 h (Lit/d DM) was higher for control, LML and LMLC than the other two diets (Table 6). The potential of gas production (A, ml/g DM), constant rate of gas production (c, h^{-1}) and lag phase were not different between treatments. Ex-vivo methane emission of diets (ml/g DM) was not different, but the variation in DMI between treatments caused the significant difference for methane emissions, whereas these values in control and LML were more than the others (P<0.05).

Table 5
Effect of CP level and rumen-protected (RP) AA supplementation on cumulative gas production and methane emission (ml/gr DM) in dairy cows

Hours	Experimental diets[1]					SEM[2]	P-Value
	Control	LM	LL	LML	LMLC		
2	74.96	74.16	78.76	78.16	79.56	2.27	0.37
4	117.68	115.28	118.48	118.88	120.68	2.57	0.67
6	153.64	149.24	153.24	152.44	154.44	2.9	0.75
8	186.52	181.32	184.92	183.52	184.52	3.33	0.85
12	224.32	218.52	223.12	221.32	221.92	3.56	0.82
24	275.56	268.16	271.36	270.96	269.36	4.34	0.79
48	302.08	295.68	298.08	299.48	294.28	4.53	0.76
72	321.76	316.36	318.36	320.56	315.96	4.27	0.83
96	330	325.36	325.16	327.16	324.76	4.54	0.92
120	334.4	328.16	327.84	329.44	328.04	4.67	0.84

Within a column, means without a common superscript letter differ (P<0.05).
n = 25; n represents number of observations used in the statistical analysis.
[1]Control = diet containing 16.2% CP; LM = diet containing 14.2% CP supplemented with RPMet Mepron [Evonik Nutrition & Care GmbH, Hanau, Germany]; LL = diet containing 14.2% CP supplemented with RPLys [Timet; VETAGRO S.p.A.; Reggio Emilia, Italy]; LML= diet containing 14.2% CP supplemented with RPLys and RPMet; LMLC= diet containing 14.2% CP supplemented with RPLys and RPMet and RPChol ReaShure (Balchem Corp., New Hampton, NY).
[2]SEM: Standard errors of mean.

Table 6
Effect of CP level and rumen-protected (RP) AA supplementation on ruminal gas production paramaters and methane emission in dairy cows

Item	Experimental diets[1]					SEM[2]	P-Value
	Control	LM	LL	LML	LMLC		
Ruminal Gas production parameters							
Total gas production in 24h, Lit/d DM	7520.0[a]	7114.3[b]	7117.8[b]	7272.6[ab]	7218.9[ab]	116.52	0.129
A[3], ml/g DM	292.95	285.64	288.80	289.05	285.50	5.150	0.839
c[4], h^{-1}	0.117	0.115	0.115	0.114	0.117	0.003	0.906
Lag[5], h	0.485	0.578	0.695	0.739	0.755	0.087	0.183
Methane emission							
CH_4 production in 24h, ml/g DM	22.05	21.18	21.44	21.57	21.25	0.345	0.440
CH_4 production in 24h, Lit/d DM	601.6[a]	562.03[b]	562.3[b]	578.9[ab]	569.6[b]	9.258	0.037

Within a row, means without a common superscript letter differ (P<0.05).
[1]Control = diet containing 16.2% CP; LM = diet containing 14.2% CP supplemented with RPMet Mepron [Evonik Nutrition & Care GmbH, Hanau, Germany]; LL = diet containing 14.2% CP supplemented with RPLys [Timet; VETAGRO S.p.A.; Reggio Emilia, Italy]; LML= diet containing 14.2% CP supplemented with RPLys and RPMet; LMLC= diet containing 14.2% CP supplemented with RPLys and RPMet and RPChol ReaShure (Balchem Corp., New Hampton, NY).
[2]SEM: Standard errors of mean.
[3]A = asymptotic gas production (ml/g DM incubated);
[4]c = fractional rate of fermentation 3(h-1);
[5]Lag = lag time (h).

Fermentable energy, being a considerable energy source for ruminants, is produced from the breakdown of lignocellulosic material by ruminal microbiome enzymes as well as gas production resulted through fermentation of carbohydrate substances (Solden et al., 2018; Stewart et al., 2018). Our results of gas production showed no significant differences among the test diets. The amount of most effective materials in gas production may include NDF, ADF, NFC and CP. The NDF and ADF were in equal amounts in experimental diets, whereas numerically the NFC and CP were higher and lower in low protein, respectively. We assumed that because the N-NH3 of the diets could prepare suitable amount of N for microorganisms growth, therefore apart from the carbohydrate structure presented in CP-source feed stuffs the N part has less influence in gas production. On the other hand, the mentioned carbohydrate structure maybe has been able to compensate for the deficiency of about 1.7% of NFC in the control diet, resulted in the equal volume of cumulative gas production.

As it has been shown in Table 6 no significant differences were detected in ruminal gas production parameters. In the rumen, an anaerobic chamber, bacteria attach to the cut edge of feed stuff to develop their colonies, this attachment needs time which is called lag phase. Lag phase was similar in experimental diets defining that the gas production started in the similar time in the rumen, however lag phase was numerically less for the control diet compared to low protein diets. Gas production parameters A (ml/g DM) and c (ml h^{-1}) didn't

differ among the diets showing that diets with reduced CP may have the similar kinetics of digestion and gas production, but the scenario of gas production is not limited to simply one gram of DM, the DMI plays an important role in gas production volume and methane emission (Hristov & Melgar, 2020). Where the total gas production of 24 h based on DMI were higher for control, LML and LMLC.

Molecular hydrogen and CO_2 are main substances for ruminal methanogen bacteria to produce methane, H2 can be produced through fermentation of carbohydrates when butyrate and acetate is produced, too (Moss, Jouany, & Newbold, 2000). Although Acetate level was increased in DMP diets, nevertheless we found no significant differences in CH4 production in 1 gr of DM of test diets. Beside the acetate and butyrate as methane promoter pathways, propionate production is assumed as an opponent (Moss et al., 2000). We found that the propionate was increased in the low protein like acetate, therefore referring to acetate: propionate ratio which was not significant among the experimental diets explaining the insignificancy of methane production. Our results agreed with those reported by Hynes et al. (2016), they reduced CP level from 18.1 to 14.1 but no significant difference in methane emission was obtained, however they have used fresh-cut grass beside the reduced level of concentrate CP. But discussing in the volume of methane production when DMI is included revealed that control diet produced much more methane than the other low protein ($P<0.05$). It was concluded that gas production values and methane emission in the farm level is not only depended on diet but also the DMI plays an important role.

Conclusions

We didn't detect any significant difference in DMI with RPAA supplementation to a dairy cattle diet being deficient in MP. Fecal parameters induced a reduction in CP and ADF digestibility in DMP diets. Adding both RPAA or with RPcholine compensated MP deficient in milk yield and protein. Nitrogen excretion as urinary N and total excreta N was significantly decreased which reduces loaded N to the environment. Ruminal pH and acetate: propionate ratio did not differ among test diets, but the ruminal ammonia-N decreased with test diets. Calculated cumulative gas production and methane emission in the farm level decreased significantly among test diets. Our findings indicated that reducing the N intake can be compensated by adding RPAA not only to keep the animal performance but also decreasing the environmental contamination by nitrogen and methane as greenhouse gases.

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