Research Square

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Nitrate and/or oils supplementation to diets with different roughage: concentrate ratios on in vitro some rumen parameters and protozoa population

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Abstract

This study aimed to determine the effect of nitrate and/or oils supplementation alone or in combination to diets with different roughage: concentrate ratios on in vitro methane (CH_4) production, volatile fatty acid (VFA) and ammonia (NH_3) concentrations, pH, and protozoa population. The experimental treatments comprised iso-nitrogenous total mixed rations based on forage with roughage to concentrate ratio of 40R:60C (High concentrate) or 60R:40C (High roughage) supplemented with 2 sources of nitrogen (sodium nitrate (NO_3^-: 45.94 g/kg DM) or urea (16.45 g/kg DM for control group) and 3 oil sources ((feed-derived oil (FDO: with no oil addition), hazelnut oil (HO: 36.58 g/kg DM) or soybean oil (SO: 36.58 g/kg DM)). For this purpose, 2 x 2 x 3 factorial design in 12 groups were used. The treatments were as follows: 40R:60C + Urea (Control) + FDO, 40R:60C + NO_3^- + FDO, 40R:60C + Urea (Control) + HO, 40R:60C + Urea (Control) + SO, 40R:60C + NO_3^- + HO, 40R:60C + NO_3^- + SO, 60R:40C + Urea (Control) + FDO, 60R:40C + NO_3^- + FDO, 60R:40C + Urea (Control) + HO, 60R:40C + Urea (Control) + SO, 60R:40C + NO_3^- + HO, 60R:60C + NO_3^- + SO. Then, the effect of nitrate, oils (O), roughage:concentrate ratio, and the combined effect of nitrate and oil associated with roughage:concentrate on in vitro methane production, VFA and NH_3 concentrations, pH, and protozoa population were evaluated. In this study, while NO_3^-, O, 40R:60C x NO_3^-, 40R:60C x O, 40R:60C x NO_3^- x O, 60R:40C x NO_3^- 60R:40C x O, 60R:40C x NO_3^- x O ($p<0.01$) decreased in vitro CH_4, protozoa population, ammonia (NH_3) concentration, acetic acid, total VFA, acetic acid: propionic acid ratio and pH, they increased butyric acid and propionic acid concentrations.

Furthermore, in vitro CH_4 production (12.44 vs 9.09 ml), NH_3 (8.23 vs 7.37 mmol/l), propionic acid (19.14 vs 17.93 mmol/l), butyric acid (15.50 vs 14.50 mmol/l), total VFA (86.46 vs 85.66 mmol/l), protozoa population (32.16 vs 26.96 x10^4 ml.) were high in the 40R:60C treatment (high concentrate). Although, 40R:60C x NO_3^- x SO, 60R:40C x NO_3^- x SO, and SO decreased more acetic acid concentration, protozoa population, and thus in vitro CH_4 production than other groups.

1. Introduction

Methane (CH_4), a product of ruminal microbial fermentation, is a major contributor to global warming (IPCC, 2014). One of the main actors in CH_4 production are ruminants. The amount of CH_4 produced in the rumen is an indicator for estimating environmental impacts and energy costs in the animal production sector (Auffret et al., 2018). Archaea are responsible for microbial fermentation in anaerobic rumen environment (Yang et al., 2017). For this reason, the decrease of the population of archaea is related to CH_4 mitigation in the rumen.

Using nitrate as a feed additive in ruminant nutrition, is one of best strategies to reduce enteric CH_4 emissions (Hristov et al., 2013). In the rumen, nitrate uses free hydrogen for the production of ammonia to the detriment of CH_4 production. Thus, CH_4 production decreases (van Zijderveld et al., 2011). Some studies show a decrease of enteric methanogenesis, microbial growth (Ungerfeld and Kohn, 2006), inhibition of total gas volume and CH_4 emission (Guyader et al., 2016), an increase of ammonia (NH_3) concentration in the rumen (Sharifi et al., 2018) due to the use of nitrate in the ration. Another CH_4 mitigation way is the use of lipids in the diet. In recent years, the use of lipids as a feed additive has been adopted as an alternative to mitigate CH_4 in the rumen (Boadi et al., 2004; Martin et al., 2010). Some researchers have reported that the alfalfa plant (because of its high nutritional value) positively affects the levels of digestion and absorption of nutrients, leading to increased productivity levels in ruminants (Paterson et al., 1982; Hunt et al., 1985; Brandt and Klopfenstein, 1986a; b; Leng 1990; Ørskov et al., 1999). On the other hand, some studies reported a low fiber content for alfalfa (28–30%) (Muir et al., 2003; Koukoura et al., 2009; Kuchenmeister et al., 2013; KanthaRaju et al., 2018). It has been found that the rations used in the present study have low fiber content. In addition, nutritional contents of rations used in both R:C ratios in the present study are similar to some studies (Sharifi et al., 2018; Alvarez-Hess et al., 2019; Villar et al., 2019; Olomonchi et al., 2019). Lipids plays an important role due to their effect on the protozoa population. In fact, protozoa stimulate hydrogen production and so methanogenesis (Guyader et al., 2015). Lloyd et al. (1989), reported rumen protozoa to have a high oxygen-scavenging ability. Thanks to these ability protozoa lead to decrease in production of H_2 and CH_4 in rumen. Here, oils rich in unsaturated fatty acids (monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA)) reduce CH_4 emissions (McGinn et al., 2004; Beauchemin et al., 2007). Some studies have shown the complementary mitigation effect of lipids and nitrate on CH_4 production in dry cows (Guyader et al., 2015) and dairy cows (Guyader et al., 2016). The aim of our study is to determine the effect of nitrate and/or oils supplementation alone or in combination to diets with different roughage: concentrate ratios on some rumen parameters and protozoa population.

2. Materials And Methods

2.1. Rations and experimental design

This study was conducted in the Laboratory of Animal Nutrition, Department of Animal Science, Faculty of Agriculture, Ondokuz Mayıs University, Samsun, Turkey. Our study was carried out according to 2 x 2 x 3 factorial design in 12 groups. The experimental treatments comprised iso-nitrogenous total mixed rations based on forage with roughage to concentrate ratio of 40R:60C (High concentrate) or 60R:40C (High roughage) supplemented with 2 sources of nitrogen (sodium nitrate (NO_3^-: 45.94 g/kg DM) or urea (16.45 g/kg DM for control group) and 3 oil sources ((feed-derived oil (FDO: with no oil addition), hazelnut oil (HO: 36.58 g/kg DM) or soybean oil (SO: 36.58 g/kg DM)). For this purpose, 2 x 2 x 3 factorial design in 12 groups were used. The treatments were as follows: 40R:60C + Urea (Control) + FDO, 40R:60C + NO_3^- + FDO, 40R:60C + Urea (Control) + HO, 40R:60C + Urea (Control) + SO, 40R:60C + NO_3^- + HO, 40R:60C + NO_3^- + SO, 60R:40C + Urea (Control) + FDO, 60R:40C + NO_3^- + FDO, 60R:40C + Urea (Control) + HO, 60R:40C + Urea (Control) + SO, 60R:40C + NO_3^- + HO, 60R:60C + NO_3^- + SO. Treatment groups and rations content were shown in Table 1. N sources (sodium nitrate and urea) and oils (hazelnut oil and soybean oil) were purchased from market. Medicago sativa was obtained from research farm of Ondokuz Mayis University in Bafra district. Rumen fluid used in this study was taken from a private slaughterhouse operating in Atakum district of Samsun. Chemical composition of rations used are given in Table 2.

Table 1
Treatment groups and rations content (g/kg DM)

Ingredients	Treatment ($g.kg^{-1}$ DM)											
	40R:60C						60R:40C					
	Control			NO_3^-			Control			NO_3^-		
	FDO	HO	SO	FDO	HO	SO	FDO	HO	SO	FDO	HO	SO
Roughage (*Medicago sativa*)	400	400	400	400	400	400	600	600	600	600	600	600
Wheat bran	140.14	140.14	140.14	140.14	140.14	140.14	128	128	128	128	128	128
Sunflower Seed Meal (SSM) (28%)	-	-	-	-	-	-	62.08	62.08	62.08	62.08	62.08	62.08
D.D.G.S(Corn)	78.6	78.6	78.6	78.6	78.6	78.6	48	48	48	48	48	48
SSM (%36)	185.29	185.29	185.29	185.29	185.29	185.29	43.06	43.06	43.06	43.06	43.06	43.06
Corn extract	60	60	60	60	60	60	30.45	30.45	30.45	30.45	30.45	30.45
Cracked wheat	42	42	42	42	42	42	28	28	28	28	28	28
SSM sieved wastes	18	24	18	24	18	24	16	16	16	16	16	16
Molasses	23.4	23.4	23.4	23.4	23.4	23.4	15.6	15.6	15.6	15.6	15.6	15.6
Corn	13.2	13.2	13.2	13.2	13.2	13.2	-	-	-	-	-	-
Sesame sieved wastes	9	9	9	9	9	9	8	8	8	8	8	8
Sesame bran	9	9	9	9	9	9	8	8	8	8	8	8
Potassium	1.06	1.06	1.06	1.06	1.06	1.06	0.41	0.41	0.41	0.41	0.41	0.41
Methionine	0.43	0.43	0.43	0.43	0.43	0.43	1.03	1.03	1.03	1.03	1.03	1.03
Lysine	0.74	0.74	0.74	0.74	0.74	0.74	0.37	0.37	0.37	0.37	0.37	0.37
Calcium	1.04	1.04	1.04	1.04	1.04	1.04	1.01	1.01	1.01	1.01	1.01	1.01
Phosphorus	0.80	0.80	0.80	0.80	0.80	0.80	0.83	0.83	0.83	0.83	0.83	0.83
Sugar	5.6	5.6	5.6	5.6	5.6	5.6	5.02	5.02	5.02	5.02	5.02	5.02
Starch	17	17	17	17	17	17	16.03	16.03	16.03	16.03	16.03	16.03
Bypass starch	7.09	7.09	7.09	7.09	7.09	7.09	8.05	8.05	8.05	8.05	8.05	8.05
Halzenut oil	-	36.58	-	-	36.58	-	-	36.58	-	-	36.58	-
Soybean oil	-	-	36.58	-	-	36.58	-	-	36.58	-	-	36.58
Sodium nitrate	-	-	-	45.94	45.94	45.94	-	-	-	45.65	45.65	45.65
Urea	16.45	16.45	16.45	-	-	-	16.34	16.34	16.34	-	-	-
UFL	83.54	83.54	83.54	83.54	83.54	83.54	81.02	81.02	81.02	81.02	81.02	81.02
UFV	79	79	79	79	79	79	76.12	76.12	76.12	76.12	76.12	76.12

FDO: Feed-derived oil (with no oil addition), Vitamin bovine A^+ (0.6; 0.4 g/kg DM), marble powder (12; 8 g/kg DM), Salt (3; 2 g/kg DM), Niacin 200 (0.6; 0.4 g/kg DM), Novatan (0.6; 0 g/kg DM), Magnesium oxide (0.6; 0.4 g/kg DM), Yeast (0.6; 0.4 g/kg DM), ME: Metabolic energy, PDIE: true protein absorbable in the small intestine, PDIN = true protein absorbable in the small intestine when degradable N is limiting microbial, UFL: Net energy form ilk, UFV: Net energy for meat.

Ingredients	Treatment (g.kg^{-1} DM)											
	40R:60C						60R:40C					
	Control			NO_3^-			Control			NO_3^-		
	FDO	HO	SO	FDO	HO	SO	FDO	HO	SO	FDO	HO	SO
PDIN (g/kg DM)	147.35	147.35	147.35	147.35	147.35	147.35	132.7	132.7	132.7	132.7	132.7	132.7
PDIE (g/kg DM)	120.96	120.96	120.96	120.96	120.96	120.96	101.25	101.2	101.25	101.25	101.2	101.2
ME (kcal. Kg^{-1} DM)	2550	2550	2550	2550	2550	2550	2500	2500	2500	2500	2500	2500

FDO: Feed-derived oil (with no oil addition), Vitamin bovine A$^+$ (0.6; 0.4 g/kg DM), marble powder (12; 8 g/kg DM), Salt (3; 2 g/kg DM), Niacin 200 (0.6; 0.4 g/kg DM), Novatan (0.6; 0 g/kg DM), Magnesium oxide (0.6; 0.4 g/kg DM), Yeast (0.6; 0.4 g/kg DM), ME: Metabolic energy, PDIE: true protein absorbable in the small intestine, PDIN = true protein absorbable in the small intestine when degradable N is limiting microbial, UFL: Net energy form ilk, UFV: Net energy for meat.

Table 2

Nutrient content of rations with different roughage:concentrate ratios supplemented with NO_3^-, O (HO and SO), and NO_3^- + O.

Nutrients (g/kg DM)	40R:60C						60R:40C					
	Control			NO_3^-			Control			NO_3^-		
	FDO	SO	HO	FDO	SO	HO	FDO	SO	HO	FDO	SO	HO
Ash	6.21	6.33	6.62	6.20	6.85	6.90	8.81	9.05	9.65	8.91	9.54	9.75
NDF	33.27	33.80	33.27	33.90	33.99	33.90	34.25	34.74	34.88	34.52	34.88	34.78
ADF	20.18	20.94	20.33	20.35	20.57	20.77	21.00	21.75	21.29	21.70	21.81	21.60
EE	3.70	8.27	8.85	3.60	9.41	9.35	3.47	9.26	9.52	3.68	9.23	9.45
CP	23.55	24.20	24.05	23.50	24.32	24.03	23.75	24.10	24.01	23.67	24.46	24.54
CF	24.85	23.12	23.00	24.60	23.12	23.63	24.32	22.38	22.46	24.10	21.86	21.33
ADL	7.63	7.90	8.10	7.55	7.79	7.74	8.95	8.25	8.50	9.02	8.60	8.75
HCEL	13.09	12.86	12.94	13.55	13.42	13.13	13.25	12.99	13.59	12.82	13.07	13.18
CEL	12.56	13.04	12.23	12.80	12.78	13.03	12.05	13.50	12.79	12.68	13.21	12.85
OM	84.23	84.00	83.77	84.50	83.80	83.56	83.96	83.90	83.36	83.05	82.96	82.68
NFE	32.13	28.41	27.90	32.80	26.95	26.55	32.42	28.06	27.37	31.60	27.41	27.30
NFC	46.36	40.26	40.70	46.35	38.85	38.95	42.97	35.84	35.53	42.04	34.96	34.66
CT (g/kg DM)	3.6	3.6	3.6	3.6	3.6	3.6	5.4	5.4	5.4	5.4	5.4	5.4
Saponin (g/kg DM)	4.28	4.28	4.28	4.28	4.28	4.28	6.42	6.42	6.42	6.42	6.42	6.42

FDO: Feed-derived oil (with no oil addition), **NDF**: Neutral Detergent Fiber, **ADF**: Acid Detergent Fiber, **EE**: Extract Ether, **CP**: Crude Protein, **CF**: Crude Fat, **ADL**: Acid Detergent Lignin, **HCEL**: Hemicellulose, **CEL**: Cellulose, **OM**: Organic Matter, **NFE**: Nitrogen Free Extract, **NFC**: Non-Fiber Carbohydrate, **CT**: Condensed Tannins.

2.2. Determination of in vitro CH_4 production

Infrared CH_4 analyzer (Sensor Europe GmbH, Erkrath, Germany model) was used to determine the in vitro CH_4 production in the rations used in present study (Goel et al., 2008). After 24 hours, the gas accumulated in the injectors was taken to the CH_4 analyzer by means of a special tube (using plastic injectors) and CH_4 production (ml) was determined as a percentage of total gas.

CH_4 production (ml) = Total gas production (ml) x % CH_4

2.3. Determination of NH_3 concentration in the rumen fluid.

For the determination of NH_3 concentration, 5 ml of rumen fluid was taken from the syringes after 48 hours. As in the protein analyses, a distillation was performed. Then the titration was done and the volume of HCl (0.1) was noted. Due to following formula, the amount of NH_3 was determined.

NH_3(mg/dl rumen fluid) = 0.1 x 14 x 1.22 (A-B) x 20

A: Volume of HCl titration solution spent in titration for the sample (ml).

B: Volume of HCl titration solution spent in titration for the witness (ml).

0.1: Normality of HCl titration solution.

14: Molar masses of nitrogen.

2.4. pH and VFA analysis in rumen fluid

In our study, 5 ml of rumen fluid was added to 2 wheaton flasks before incubation and 4 drops of H_2SO_4 were added to determine the content of VFA in the rumen fluid to be used in the study. The rumen fluids thus prepared were kept at room temperature until the analyzes were performed. The rumen fluid taken at 48th hour was subjected to the same treatment. The pH of the rumen liquid used in the experiment was determined by digital pH meter (HANNA INSTRUMENTS 1332 model pH meter) as soon as it was brought to the laboratory. Rumen fluid taken at 48th hour was subjected to the same treatment. VFA content of the ruminal fluid (Obtain after 48th hour of incubation) was made using the procedure described by Wiedmeier et al. (1987) and using gas chromatography (Agilent Tech. 6890N GC, Stabilwax-DA, 30 m, 0.25 mm ID, 0.25 µm df. Max. Sıcaklık: 260°C. Cat. 11023) at the University of Uludağ, Faculty of Agriculture, Department of Animal Sciences. Four drops of sulfuric acid were added to about 5 ml of ruminal liquid fluid and the mixture was maintained at -20°C and centrifuged at 10000 rpm at + 4°C.

2.5. Determination of Protozoa Population

After the mixing 0.6 g methyl green, 8 g sodium chloride (NaCl) and 100 ml 37% formaldehyde solution for staining the protozoa, the volume was increased to 1000 ml with distilled water. One mililiter of rumen inoculum from the fermenter was mixed with 1 ml of methyl-green-formalin solution (MFS). The protozoa number was carried out with the object slide of a light microscope and Fuchs-Rosenthal counting chamber (depth:0.2 mm, small square area: 0.0625 mm^2) (Ranilla et al., 1997). This mixture (Rumen fluid + MFS) was kept at -20°C until analysis time. Subsequently, the samples taken from this mixture and shaken were placed on a Fuchs-Rosenthal slide (16 x 16 squared. 0.0625 mm ~ area). Calculation is made with the formula given below:

$$\mathbf{Numberofcellsincm}^3\,(\mathrm{ml}) = 1000\mathbf{x}\frac{\mathbf{Numberofcellscounted}}{\mathbf{TotalframescountedxDilutionxVolume}}$$

2.6. Statistical analysis

Data obtained as a result of the research (in vitro CH_4 production, NH_3 concentration, volatile fatty acids (VFA), pH, and protozoa population) were checked for the necessary assumptions (such as normality and homogeneity of variances) and then analyzed in a randomized plot according to factorial experiment. The following model was used in the study.

$Yijkl = \mu + \alpha_i + \beta_j + \lambda_k + (\alpha\beta)ij + (\beta\lambda)_{jk} + (\alpha\lambda)_{ik} + (\alpha\beta\lambda)_{ijk} + e_{ijkl}$

Where Y_{ijkl}: i^{th} application subject to j^{th} feed variety (CH_4 production, etc.) k^{th} observation value of the sample (gas production, etc.).

µ: mean population,

α_i: Effect of i^{th} ration

βj: Effect of the j^{th} additive

λ: Effect of the k^{th} oil addition

$(\alpha\beta)_{ij}$: Interaction of i^{th} ration and j^{th} additive effect

$(\beta\lambda)_{jk}$: Effect of interaction between j^{th}inci additive with k^{th} oil type

$(\alpha\lambda)_{ik}$: Effect of interaction between i^{th} ration with k^{th} vegetable oil type

$(\alpha\beta\lambda)_{ijk}$: Effect of interaction between i^{th} ration, j^{th} additive with k^{th} oil type

eijk: shows a random error.

Duncan Multiple Comparison test was used to compare the means if the differences between the applications or feed types were statistically significant. SPSS 22.0 statistical package program licensed by Ondokuz Mayıs University was used for statistical analysis.

3. Results

3.1. In vitro methane (CH_4) production

In current study, R:C ratio ($p = 0.002$), NO_3^- ($p < 0.001$), oils addition (O) ($p < 0.001$), R:C ratio x NO_3^- interaction ($p < 0.001$), NO_3^- x O interaction ($p = 0.007$), R:C ratio x O interaction ($p < 0.005$), and R:C ratio x NO_3^- x O interaction ($p = 0.004$) were found to affect in vitro CH_4 production (Table 3). After 24 hours of fermentation, the high-concentrate (40R:60C) content rations led to higher in vitro CH_4 production. 40R:60C x NO_3^-, 60R:40C x NO_3^-, 40R:60C x NO_3^- x O, 60R:40C x NO_3^- x O treatment groups decreased in vitro CH_4 production. But, 40R:60C x SO and 60R:40C x SO treatment groups led to lower in vitro CH_4 production (compared to 40R:60C x HO and 60R:40C x HO treatment groups).

Table 3

Effects of NO_3^-, O (HO and SO), and NO_3^- + O supplemented to different roughage:concentrate ratios on rumen fermentation properties.

Roughage: Concentrate	Feed additive	Oils	CH_4	NH_3	VFA				AA:PA	PP	pH
					AA	PA	BA	TVFA			
40R:60C	Control	FDO	13.29^{a}	9.20^{a}	49.57^{b}	17.47^{d}	15.51^{bc}	89.11^{a}	2.84^{b}	36.19^{a}	6.12^{bc}
		SO	11.05^{c}	8.33^{b}	46.04^{d}	19.19^{b}	14.53^{def}	85.65^{de}	2.40^{fg}	29.25^{e}	5.95^{ef}
		HO	12.34^{b}	8.98^{a}	48.44^{bc}	18.58^{c}	14.68^{de}	87.63^{bc}	2.61^{c}	32.69^{c}	5.99^{def}
	NO_3^-	FDO	11.94^{b}	8.35^{b}	47.29^{cd}	19.21^{b}	16.89^{a}	87.14^{bcd}	2.46^{def}	35.06^{b}	6.08^{c}
		SO	7.18^{g}	6.60^{f}	43.24^{e}	20.84^{a}	15.62^{bc}	83.37^{f}	2.07^{i}	27.69^{g}	5.94^{f}
		HO	8.62^{e}	7.93^{c}	46.55^{d}	19.57^{b}	15.74^{b}	85.70^{de}	2.38^{g}	32.06^{d}	5.98^{de}
60R:40C	Control	FDO	12.27^{b}	8.34^{b}	52.49^{a}	16.77^{e}	13.95^{fg}	88.51^{ab}	3.13^{a}	28.31^{f}	6.25^{a}
		SO	7.29^{g}	7.28^{d}	46.86^{d}	18.55^{c}	13.82^{g}	84.39^{ef}	2.53^{cd}	26.81^{h}	6.00^{d}
		HO	9.15^{e}	7.96^{c}	49.36^{b}	17.31^{de}	14.07^{fg}	86.35^{cd}	2.85^{b}	27.19^{g}	6.08^{c}
	NO_3^-	FDO	9.82^{d}	7.37^{d}	51.19^{ab}	16.80^{e}	15.70^{b}	89.93^{a}	3.04^{ab}	28.06^{f}	6.16^{b}
		SO	7.35^{f}	6.37^{f}	43.85^{e}	19.66^{b}	14.41^{efg}	81.78^{g}	2.23^{h}	25.36^{j}	5.99^{de}
		HO	8.68^{c}	6.87^{e}	45.97^{d}	18.47^{c}	15.04^{cd}	83.01^{fg}	2.49^{de}	26.06^{i}	6.08^{c}
Roughage:Concentrate (R:C)											
40R:60C			12.44	8.23	46.86	19.14	15.50	86.43	2.46	32.16	6.01
60R:40C			9.09	7.37	48.45	17.93	14.50	85.66	2.72	26.96	6.09
Feed additives (FA)											
Control			9.57	8.35	48.79	17.98	15.28	86.94	2.72	30.57	6.07
NO_3^-			8.62	7.25	46.52	19.09	14.71	85.15	2.46	29.97	6.04
Oils											
FDO			11.83	8.32	50.39	17.56	15.52	88.67	2.88	31.91	6.15
SO			8.22	7.15	45.00	19.56	14.60	83.79	2.31	27.28	5.97
HO			9.70	7.94	47.58	18.48	14.88	85.67	2.58	29.50	6.04
S.E.M.			0.306	0.149	0.481	0.207	0.155	0.429	0.54	71.559	0.015
Means effects											
R:C			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
NO_3^-			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.006
Oils			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

a. b. c… The averages shown with different letters in the same column are different from each other.

CH_4: Methane (ml) NH_3: Ammonia (mg/dl), AA: Acetic acid (mmol/l), PA: Propionic acid (mmol/l), BA: Butiric acid (mmol/l), PP: Protozoa Population (x10^4 ml), NO_3^-: nitrate, HO: hazelnut oil, SO: soybean oil, S.E.M: Standard error of means, **FDO**: Feed-derived oil (with no oil addition).

Roughage: Concentrate	Feed additive	Oils	CH_4	NH_3	VFA				AA:PA	PP	pH
					AA	PA	BA	TVFA			
F:C × NO_3^-			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.031	0.013
F:C × Oils			< 0.001	0.008	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.033
NO_3^- × Oils			0.015	0.004	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.004	0.008
F:C × NO_3^- × Oils			< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.017	0.015

a. b. c... The averages shown with different letters in the same column are different from each other.

CH_4: Methane (ml) NH_3: Ammonia (mg/dl), AA: Acetic acid (mmol/l), PA: Propionic acid (mmol/l), BA: Butiric acid (mmol/l), PP: Protozoa Population (x10^4 ml), NO_3^-: nitrate, HO: hazelnut oil, SO: soybean oil, S.E.M: Standard error of means, **FDO**: Feed-derived oil (with no oil addition).

3.2. NH_3 concentration in the rumen fluid.

After 48 hours of incubation, R:C ratio ($p < 0.001$), NO_3^- ($p < 0.001$), O ($p < 0.001$), R:C x NO_3^- ($p < 0.05$), R:C ratio x O ($p < 0.05$), NO_3^- x O ($p < 0.05$) and R:C ratio x NO_3^- x O ($p < 0.05$) affected NH_3 concentration. In the present study, it was found that NH_3 concentration ($p < 0.001$) increased as concentrate level increased. But, 40R:60C x NO_3^-, 60R:40C x NO_3^-, 40R:60C x NO_3^- x oil, 60R:40C x NO_3^- x oil treatment groups led to the lowest NH_3 concentration. Especially 40R:60C x NO_3^- x SO and 60R:40C x NO_3^- x SO treatment groups were found to have the lowest NH_3 concentration. By the way, 40R:60C x urea$^-$ x O and 60R:40C x urea x O treatment groups (control groups) had higher NH_3 concentration.

3.3. pH, VFA, and AA: PA ratio

The pH values, concentration of total fatty acids (TVFA), AA, PA, BA, and AA:PA ratio were affected by R:C ratio, NO_3^-, O, and R:C ratio x FA, R:C ratio x O, FA x O, R:C ratio x FA x O interactions (Table 3). In this study, 40R:60C (high concentrate) increased PA, BA, TVFA concentrations. However 60R:40C (high roughage) increased AA, AA:PA ratio and pH. By the way, 40R:60C x NO_3^-, 60R:40C x NO_3^-, 40R:60C x NO_3^- x oil, 60R:40C x NO_3^- x oil treatment groups ($p < 0.001$) increased PA and BA concentration, but decreased AA concentration, TVFA, AA:PA ratio, and pH ($p < 0.001$).

3.4. Protozoa population

Protozoa number was affected by R:C ratio ($p < 0.001$), NO_3^- ($p < 0.001$), O ($p < 0.001$), R:C ratio x NO_3^- ($p < 0.05$), R:C ratio x O ($p < 0.001$), NO_3^- x O ($p < 0.05$) and R:C ratio x NO_3^- x O interaction ($p < 0.05$). 40R:60C (high concentrate) increased protozoa number. But compared to 60R:40C (high roughage) decreased protozoa number. At the end of 48 hours of fermentation, 40R:60C x NO_3^-, 60R:40C x NO_3^-, 40R:60C x NO_3^- x oil, 60R:40C x NO_3^- x oil treatment groups decreased protozoa population more than other groups ($p < 0.05$). The decreasing effect of NO_3^- and Oils on protozoa population was more evident with 40R:60C x NO_3^- x SO and 60R: 40C x NO_3^- x SO treatment groups.

4. Discussion

4.1. CH_4 production

A low in vitro CH_4 production recorded in 60R:40C (high roughage) may be associated with the presence of high levels of secondary metabolites (saponin and condensed tannin) in *Medicago sativa* used as a roughage in this study (Kozlowska et al., 2020). Previously, Castro-Montoya et al. (2012) and recently Kozlowska et al. (2020) found that the plantes rich in saponin (quillaja plant, alfalfa or *Medicago sativa*) used in high level in diet (high roughage for example) decreased CH_4 production compared to high concentrate. Likewise, in some studies, saponin has been found to reduce CH_4 production (Morgavi et al., 2012; Jayanegara et al., 2014; Chen et al., 2019). A low in vitro CH_4 production found in high roughage and high roughage can be explained by low NDF and high CP of all of diets used in this study (Table 2). However a lowest in vitro CH_4 production found in a high roughage (60R:40C) compare to the high concentrate (40R:60C) is due to the presence of saponin and condensed tannins (in high level, Table 2) and the combined effect of secondary metabolites (Saponin and

Condensed tannins), NO_3^- and oils. While some researchers found that a low condensed tannin (CT < 0.001%) content in alfalfa hay decreased CH_4 production, some researchers reported a decrease in CH_4 production due to the low NDF and a high crude protein content and a presence of secondary metabolites in alfalfa (Cheok et al., 2014; Rira et al., 2015; Moate et al., 2017; Szumacher -Strabel et al., 2019). These reports are consistent with our findings.

In current study, the effect of NO_3^- supplementation on in vitro CH_4 production in the high concentrate and high roughage was different. In this study the increase of roughage level (60R:40C) led to a decrease of in vitro CH_4 production. This can be explained by the presence of secondary metabolites (Saponin and Condensed tannin) and the effect of nitrate which act to reduce in vitro CH_4 production. Previously an interaction was found between ration type (roughage/concentrate ratio) and CH_4 reducing agents (such as nitrate) in cattle (Alvarez-Hess et al., 2019). This result is consistent with our findings.

In vitro CH_4 production was high when a concentrate ratio was high in the diet and was gradually decrease with the high roughage ration in the diet. This could be due to that roughage (Medicago sativa) content a high secondary metabolites (Saponin and condensed tannin) which could explain the lower in vitro CH_4 production when roughage fraction was maximun (60%) in the diets. A high in vitro CH_4 production found in a high concentrate level, is consistent with some studies (Hristov et al., 2015; 2017; Moate et al., 2017; 2019; Olomonchi et al., 2022). In this study, a relationship between in vitro CH_4 production, dietary starch rate and digestion can be established. This is in line with previous findings (Herrera-Saldana et al., 1990; McAllister et al., 1996; Alvarez-Hess et al., 2019).

In our study, it was found that NO_3^- + O added rations decreased in vitro CH_4 production in the high concentrate (40R:60C) and high roughage (60R:40C) ($p < 0.001$). Some researchers reported that a combined effect of oils (rich MUFA or PUFA) and NO^-_3 is an effective method to reduce CH_4 production in rumen (Leng and Preston, 2010; Yang et al., 2016). It has been determined that a lower effect of NO_3^- on in vitro CH_4 production is associated to NO_3^- and nitrite reducing microorganisms (Guo et al., 2009). Nitrate acts as a hydrogen acceptor. There are studies showing that nitrate has a significant inhibitory effect on CH_4 production (El-Zaiat et al., 2014; Olijhoek et al., 2016). In the current study, the reduction of nitrate to nitrite and then to NH_3 reduces H ions concentration required for the conversion of the CO_2 to CH_4 compound in the rumen and thus in vitro CH_4 production decreases. Previously some studies reported the same result (Zhou et al., 2012; Liu et al., 2017). In addition, it has been determined that nitrite has a toxic effect on methanogens (Božic et al., 2009; Zhou et al., 2011). However, in this study the reducing effect of NO_3^- on CH_4 is more evident in rations with a high roughage which is rich in condensed tannins or saponins. Similar results have been reported from an experiment by Pal et al. (2014).

In our study, while a high in vitro CH_4 production was observed in 40R:60C (12.44 ml), a lower CH_4 production (9.09 ml) was recorded in low a high roughage level (Table 3). This result is related to the increase in saponin and condensed tannins level and their effects on CH_4 production in a high roughage level.

In our study, oils used were rich in PUFA (SO) and MUFA (HO). As expected, SO with a high PUFA content decreased in vitro CH_4 production at a higher level than HO. This finding is consistent with studies reporting that the mitigation effect of oils on CH_4 production is related to degree of unsaturation (Rodrigues et al., 2017; Vargas et al., 2017).

In high concentrate and high roughage, the higher negative effect of SO (rich in PUFA) in vitro CH_4 production (compared to the effect of HO) is associated with a high presence of α-linolenic acid (C18:3 cis-9, cis-12, cis-15) and linoleic acid (C18:2 cis-9, cis-12) in SO. Previously, the effect of oils such as flaxseed and rapeseed rich in PUFA on CH_4 mitigation was found by some researchers (Chung et al., 2011; Benchaar et al., 2015; Veneman et al., 2015). As a matter of fact, a lowering effect of oils rich in MUFA (oleic acid (C18:1)) on CH_4 mitigation was found (Dong et al., 1997). Although, in the high concentrate and high roughage, HO decreased in vitro CH_4 production. Likewise, in some studies canola oil (22% linoleic acid, 11% linolenic acid, and 54% oleic acid) caused a reduction of CH_4 production (Dohme et al., 2000; Beauchemin and McGinn, 2015)). It was found that oil (rich in MUFA or PUFA) reduced the cellulolytic bacteria population, methanogenic bacteria, and then CH_4 production (Freitas et al., 2018; Nur Atikah et al., 2018).

In present study, the decrease in CH_4 production due to oils addition can be associated with the decrease in protozoa population. In the high concentrate and high roughage, it was determined that NO_3^- + O supplementation caused a higher decrease in CH_4 production compared to the use of O and NO_3^- separately. This result is consistent with previous studies (Duthie et al., 2017; Villar et al., 2019). Likewise, Guyader et al. (2015) and Veneman et al. (2015) reported that CH_4 production decreases when nitrate and flaxseed oil (high in MUFA) are added to ration.

4.2. NH_3 concentration

In the current study, rumen NH_3 values determined for rations used in high concentrate and high roughage are above the recommended minimum NH_3 concentration (4.39 to 7.32 mmol/l) (Satter and Slyter, 1974), which is considered sufficient for maximum microbial growth rates. The high NH_3 concentration found in the high concentrate (40R:60C) might be related to the high number of proteolytic bacteria in rumen. Because, proteolytic bacteria increase ruminal NH_3 concentration by accelerating protein degradation in rumen. While this finding is in agreement with some studies (Kljak et al., 2017; Liu et al., 2019), it disagreed with other studies (Jadhav et al., 2017; Liu et al., 2018).

A high roughage (60R:40C) decreased NH_3 concentration in present study. This finding can be associated with a high level of alfalfa (rich in saponins), which increased saponin level in ration. Saponin decreased or inhibited NH_3 production. Our results were consistent with some previous studies (Belanche et al., 2016; Jadhav et al., 2018).

In our study, 40R:60C x NO_3^- and 60R:40C x NO_3^- treatment groups decreased NH_3 ($p < 0.001$). NO_3^- is converted to nitrite, which has a toxic effect on rumen bacteria, and therefore NO_3^- addition reduces NH_3 concentration at a high level compared to urea addition (control group). Nitrate alters the fermentation profile and decreases the NH_3 production. However, the conversion rate of NO_3^- to NH_3 in rumen is slower than urea to NH_3. This can explained the high NH_3 concentrattions found in control groups (urea supplementation) compared to other treatment groups.

Various studies investigated the effect of O (rich in MUFA or PUFA) supplementation on NH_3 concentration. While in some studies oil supplementation had no effect (Jalc et al., 2005) on NH_3 concentration, in some studies oil supplementation increased (Jalc et al., 2002) or decreased (Szumacher-Strabel et al., 2009; Doreau et al., 2017) NH_3 concentration.

In this study, oils rich in PUFA (SO) or in MUFA (HO) associated with the R:C ratios (40R:60C, and 60R:40C) decreased NH_3 concentration ($p < 0.001$). This can be explained by the presence of linolenic acid (SO) and oleic acid (HO). But the effect of SO (rich in PUFA) was more evident. In fact, the biohydrogenation of linoleic acid consumes more hydrogen (compare to biohydrogenation of oleic acid). Thus, in our study, the lack of hydrogen causes the decrease in NH_3 production. Previously, while Bayat et al. (2017), and Kubelkova et al. (2018) found that flaxseed oil (rich in PUFA) compared to rapeseed oil (rich in MUFA) decreased the rumen pH and NH_3 concentration at a high level, some researchers reported that *Moringa oleifera* oil rich in MUFA (oleic acid (74.99%), stearic acid (2.09%), linolenic acid (1.75%), and linoleic acid (1.27%)) increased rumen protected (by-pass) protein and decreased NH_3 concentration (Gassenschmidt et al., 1995; Belewu et al., 2014).

In our study, a combined effect of NO_3^- + O supplementation link to the high concentrate or high rougahge decreased NH_3 concentration ($p < 0.05$). However, combined effect of NO_3^- + SO (compared to NO_3^- + HO) was more evident on NH_3 concentration in high concentrate or high roughage. In the same time, the biohydrogenation (due to O supplementation) and hydrogen sink reaction (due to NO_3^- supplementation) were happened to use the free hydrogen in rumen. Like that, NH_3 production decreased because of lack of hydrogen. Previously, combined effect of NO_3^- + O supplementation was reported in some studies (Veneman et al., 2015 (NO_3^- + linseed oil supplementation); Villar et al., 2019 (NO_3^- + canola oil supplementation)).

4.3. pH, VFA, and AA: PA ratio

In the present study, pH values of rations used, are determined from the fluids remaining in the injectors after 48 hours of incubation. The pH values vary between 5.99 and 6.25 (Table 3). The pH difference in this study is due to R:C ratio. In this study while a high concentrate decreased pH, a high roughage increased pH. Firstly, this could be to that a high roughage (60R:40C) contained more NDF, ADF and cellulose than a high concentrate (40R:60C). Secondly, a decreased in pH due to the high concentrate can be associated to a high starch content which creates an environment to inhibit nitrate and nitrite metabolism. This means that a high concentrate provided sufficient energy for the microorganisms to convert nitrate to nitrite and then nitrite to NH_3. For this reason, NH_3 concentration was high in the high concentrate (Table 3).

Although, it was found that NO_3^- addition link to R:C ratios decreased pH values ($p < 0.05$). This finding is in agreement with some studies (Li et al., 2012; Villar et al., 2019). Likewise, rumen pH values found in our study are consistent with the value reported by Latham et al. (2016). A decline in pH observed due to NO_3^- supplementation indicates that microorganisms were not accustomed to digesting nitrate. It suggested that NO_3^- supplementation caused a dramatic change in rumen conditions.

In the present study, oil addition associated to R:C ratios decreased pH values. A decrease in ruminal pH, AA concentration, and CH_4 production observed due to oil addition in our study can be associated with the degree of unsaturation of oils used (SO and HO). Some researchers have reported that oils addition reduces ruminal pH, AA concentration, and CH_4 production with oils (rich in MUFA or in PUFA) supplementation (Wu et al., 2016; Majewska et al., 2017; Alvarez-Hess et al., 2019). However, it was found that SO (compared to HO)

decreased significantly pH, AA concentration, and CH_4 production (Compared to HO). This can be associated to the high level of linolenic acid in SO. This result is consistent with some studies (Russell and Wilson, 1996; Mertens, 1997). By the way, NO_3^- + O supplementation combine with R:C ratios decreased more pH, AA concentration, and CH_4 production. This is associated to the biohydrogenation of unsaturated fatty acids (PUFA, and MUFA) provided by oil (SO, and HO), and hydrogen sink reaction (due to NO_3^- supplementation) which occurred in the same time.

In this experiment, TVFA, individual concentration of VFA (AA, PA, BA, and AA: PA ratio) were affected by R:C ratios, NO_3^-, O, R:C ratios x NO_3^-, R:C ratios x O, NO_3^- x O.

It was determined that a high concentrate decreased AA concentration, and AA: PP ratio ($p < 0.001$). An increase in PA, BA, TVFA concentration found in the high concentrate can be explained by the lowering pH due to the increase in lactic acid content derived from the high easily fermentable carbohydrates content of rations used, and an increase in carbohydrate fermentation. An increase in BA concentration can be also associated with the increase in ammonia concentration which inhibited bacterial growth and promotes a fermentation for BA production in this study. As it is known, VFA are produced as a result of microbial fermentation of carbohydrates in the rumen. However, the increase in AA, TVFA, and AA:PA ratio found in the high roughage, is associated with the increase in fiber content (in this case NDF and ADF). Depending on an increase in fibrous content of ration, ruminal hydrogen concentration used in the production of AA and in vitro CH_4 increased. Our results are consistent with some studies (Kljak et al., 2017; Moate et al., 2017; Alende et al., 2019).

The increase in PA concentration due to NO_3^- addition can be explained by the competition between the mechanism of PA production and nitrate (for ammonia production). In other words, propionic acid producing bacteria population (*Selenomonas ruminantium*, *Propionibacterium* and *Tessaracoccus*) increased and they used free H ions present in the rumen to produce propionic acid. For this reason, hydrogen required for nitrate reduction (nitrite then ammonia) decreased. Consequently, PA concentration increased and NH_3 concentration, AA and AA: PA ratio decreased in the rumen. But, a decrease in BA (due to NO_3^- supplementation) was caused by the rapid reduction of NO_3^- (to nitrite then ammonia) which use up the electrons needed for the production of BA. However, a high roughage level decreased BA concentration. This was due to the combined effect of tannin and NO_3^-. Our results were consistent with some studies (van Zijderveld et al., 2011; Adejoro and Hassen, 2017; Wang et al., 2018).

In this study, the decrease in AA due to NO_3^- supplementation can be explained by the use of free hydrogen for production of NH_3 and PA. Like this, hydrogen concentration required for the production of AA decreased. One of the possible reasons for the reduction in the concentration of AA due to the combined effect of NO_3^- and the two types of oil (MUFA or PUFA) is the use of free hydrogens for the production of PA and BA.

In our study, the effect of NO_3^- + O on VFA and AA: PA ratio changed according to the source of fatty acids (MUFA and PUFA). For that, NO_3^- + SO (rich in PUFA) associated with R:C ratios decreased AA concentration but it increased PA and BA concentrations. This result can be explained by the simultaneous effect of NO_3^- (hydrogen sinks) and the biohydrogenation of PUFA which consume more free hydrogen than the biohydrogenation of MUFA. A high concentrate level increased more the combined effect of NO_3^- + SO on AA, CH_4, NH_3, TVFA and AA:PA ratio. However, while NO_3^- stimulated the population of propionic acid-producing bacteria, the unsaturated fatty acids (PUFA and MUFA) in SO and HO used free hydrogens for biohydrogenation. Thus, the production of AA, CH_4, NH_3, TVFA and AA:PA ratio decreased. Our findings are in conformity with those found by Popova et al. (2017) and Villar et al. (2019). Our results showed that AA and CH_4 were more decreased due to the combined effect of NO_3^- + SO which can be explained by biohydrogenation of PUFA and NO_3^- mechanism (transformation of NO_3^- to nitrite then to NH_3) for obtaining PA having different and associative mechanism for using available hydrogen. Use of NO_3^- and O in the same time in the ration led to reduction in ruminal hydrogen concentration.

4.4. Protozoa population (PP)

In the current study, a high concentrate level increased the number of PP compared to the high roughage ($p < 0.001$). Previously, it was shown that a high concentrate can increase (Franzolin and Dehority, 1996; Lengowski et al., 2016) or decrease (Gozho et al., 2005; Khafipour et al., 2009; Hook et al., 2011) protozoa population.

However, the increase in roughage (rich in secondary metabolites: saponins and tannins) content of ration link to NO_3^- addition caused a decrease in the protozoa number. This can be explained by the combine effect of NO_3^- and saponins which acted negatively on protozoa population. Our findings are consistent with those of Lin et al. (2013).

In the present study, NO_3^- added rations associated with R:C ratios decreased PP. Otherwise, nitrite which come from a transformation of nitrate, inhibits rumen protozoa population and thus in vitro CH_4 production. This is consistent with findings of Iwamoto et al. (2001).

Furthermore, in our study, there is a parallelism between NH_3 concentration and PP in a high roughage level, and this finding was reported by some studies (Hu et al., 2005; Liu et al., 2018).

In our study, use of NO_3^- alone or in combination with HO (rich in MUFA: oleic acid) and SO (rich in linolenic acid and linoleic acid) reduced protozoa population. However, the combined effect of NO_3^- + SO decreased protozoa population more than individual use of NO_3^- and O. This can be explained by the simultaneous mitigation effect of NO_3^- and PUFA (linolenic acid and linoleic acid) on PP. Previously it was demonstrated that NO_3^- alone (Sar et al., 2005; Asanuma et al., 2015) or in combination with linseed oil (Veneman et al., 2015) or canola oil (Villar et al., 2019) decreased PP. While some authors notified a toxic effect of NO_3^- and lipids on protozoa PP (Morgavi et al., 2010), other researchers reported no significant effect on PP (Guyader et al., 2016).

Conclusion

In this study, a high roughage (because of a presence of saponins and condansed tannins in high level) decreased CH_4, AA, NH_3, TVFA, PP, and AA:PA ratio. While the 60R:40C associated to NO_3^-, O alone or in combination decreased CH_4, AA, TVFA, PP, and AA:PA ratio, it increased PA, BA. This study shows that NO_3^- and O (HO and SO) affect in vitro CH_4 production, protozoa population, NH_3 and VFA concentrations. The combination of NO_3^- and O (HO and SO) reduced acetic acid, protozoa population (thus in vitro CH_4), and increased propionic acid and butyric acid more than individual use of nitrate and oils. Our study showed that a combined effect of nitrate and oils can be considered more avantageous to reduce methane production and protozoa population without a negatif effect on PA and BA concentrattions.

The fermentation properties of rations supplemented with nitrate or oils have a potential to improve rumen fermentation. It has been found that the degree of unsaturated fat alone or in combination with NO_3^- decreases CH_4 production and increases VFA.

Declarations

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Conflit of interests

The authors declare that they have no conflict of interest.

Ethics Approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material (data transparency)

Data on the parameters that were the subject of this study are available from the corresponding author on reasonable request.

Code availability (Software application or custom code)

Not applicable

Author's contributions

Euloge O.A. OLOMONCHI and Ali V. GARIPOGLU conceived and designed the present study. Euloge O.A. OLOMONCHI conducted the literature search, analysed, interpreted data, and drafted the manuscript. The study was supervised by Euloge O.A. OLOMONCHI and Ali V. GARIPOGLU. All authors read and approved the final manuscript.

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