Effect of Nitrate, Two Oils and Their Combinations Added To Two Different Forage: Concentrate Ratios On Some Rumen Parameters and Protozoa Population.

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Research Article

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Abstract

This study aimed to determine the effects of nitrate, and two oils (O) (soybean and hazelnut oils) alone or in combination, on in vitro methane (CH_4) production, volatile fatty acid (VFA) and ammonia (NH_3) concentrations, pH, and protozoa population. For that, $2 \times 2 \times 3$ factorial design with 2 two different forage:concentrate (F:C) ratios (40F:60C, and 60F:40C), 2 sources of nitrogen (sodium nitrate (NO_3^- : 45.94 g/kg DM) or urea (16.45 g/kg DM for control group) and 2 oils (hazelnut oil (HO: 36.58 g/kg DM) or soybean oil (SO: 36.58 g/kg DM)). For every forage: concentrate (F:C) ratio, six (6) treatment groups were formulated: F:C + Urea, F:C + NO_3^- , F:C + Urea + HO, F:C + Urea + SO, F:C + NO_3^- + HO, F:C + NO_3^- + SO. For every F:C ratio while NO_3^- ($p < 0.01$), oils ($p < 0.01$), and NO_3^- + oils (SO, HO) ($p < 0.01$) decreased CH_4 content, 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, CH_4 production (12.44 vs 9.09 ml), ammonia (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 26.96×10^4 ml.) were high in early lactation period. In both lactation periods, SO, and NO_3^- + SO decreased acetic acid concentration, protozoa population, and thus CH_4 production.

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 both forage:concentrate ratios. In addition, nutritional contents of rations used in both forage:concentrate ratios in the present study are similar to some studies (Sharifi et al., 2018; Alvarez- Hess et al., 2019; Villar 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 methane 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 NO_3^- 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 combined effect of NO_3^- and oils (Soybean (SO) and Hazelnut (HO) oils) addition in Medicago sativa-based rations on CH_4 production and some rumen parameters (pH, NH_3 , protozoa numbers and volatile fatty acids (VFA)).

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 $2 \times 2 \times 3$ factorial design with 2 forage:concentrate ratios (40F:60C and 60F:40C), 2 sources of nitrogen (sodium nitrate (NO_3^- : 45.94 g/kg DM) or urea (16.45)) and 2 oils (Hazelnut oil (HO: 36.58 g/kg DM) or Soybean oil (SO: 36.58 g/kg DM)). Isonitrogenic rations were used in our study (urea was used in the control group in order to balance the protein content of rations). For every forage:concentrate ratio, six (6) treatment groups were formulated: F:C + Urea, F:C + NO_3^- , F:C + Urea

+ HO, F:C + Urea + SO, F:C + NO₃⁻ + HO, F:C + NO₃⁻ + 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 Mayıs 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)											
	40F:60C						60F:40C					
	Control			NO ₃ ⁻			Control			NO ₃ ⁻		
	Oil-free	HO	SO	Oil-free	HO	SO	Oil-free	HO	SO	Oil-free	HO	SO
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
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)											
	40F:60C						60F:40C					
	Control			NO ₃ ⁻			Control			NO ₃ ⁻		
	Oil-free	HO	SO	Oil-free	HO	SO	Oil-free	HO	SO	Oil-free	HO	SO
UFV	79	79	79	79	79	79	76.12	76.12	76.12	76.12	76.12	76.12
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
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 forage:concentrate ratios supplemented with NO₃⁻, O (HO amd SO), and NO₃⁻ + O.

Nutrients (g/kg DM)	40F:60C						60F:40C					
	Control			NO ₃ ⁻			Control			NO ₃ ⁻		
	Oil-free	SO	HO	Oil-free	SO	HO	Oil-free	SO	HO	Oil-free	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
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 CH₄ production

Infrared CH₄ analyzer (Sensor Europe GmbH, Erkrath, Germany model) was used to determine the CH₄ 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 CH₄ analyzer by means of a special tube (using plastic injectors) and CH₄ production (ml) was determined as a percentage of total gas

$$\text{CH}_4 \text{ production (ml)} = \text{Total gas production (ml)} \times \% \text{CH}_4$$

2.3. Determination of NH₃ concentration in the rumen fluid.

For the determination of NH₃ 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 NH₃ was determined.

$$\text{NH}_3(\text{mg/dl rumen fluid}) = 0.1 \times 14 \times 1.22 (\text{A-B}) \times 20$$

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 H₂SO₄ 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 mm²) (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:

$$\text{Number of cells in cm}^3 (\text{ml}) = 1000 \times \frac{\text{Number of cells counted}}{\text{Total frames counted} \times \text{Dilution} \times \text{Volume}}$$

2.6. Statistical analysis

Data obtained as a result of the research (in vitro CH₄ production, NH₃ 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.

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

Where Y_{ijkl}: ith application subject to jth feed variety (CH₄ production, etc.) kth observation value of the sample (gas production, etc.).

μ: mean population,

α_i : Effect of i^{th} ration

β_j : 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} additive with k^{th} vegetable 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} vegetable oil type

e_{ijk} : 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. Methane (CH₄) production

In current study, F:C ratio ($p=0.002$), feed additive (FA) ($p<0.001$), oils addition (O) ($p<0.001$), F:C ratio x FA interaction ($p<0.001$), FA x O interaction ($p=0.007$), F:C ratio x O interaction ($p<0.005$), and F:C ratio x FA x O interaction ($p=0.004$) were found to affect CH₄ production (Table 3). After 24 hours of fermentation, the high-concentrate (40F:60C) content rations led to higher CH₄ productions. In 40F:60C and 60F:40C, NO₃⁻ supplementation (compared to urea supplementation) and NO₃⁻ + O supplementation (compared to urea + O supplementation) decreased CH₄ production. This combined effect of NO₃⁻ + oils supplementation on CH₄ production was more evident with a high forage content (60F:40C) compared to high concentrate content (40F:60C). However, in 40F:60C and 60F:40C, SO supplementation led to lower CH₄ production compared to HO supplementation).

Table 3

Effects of NO_3^- , O (HO and SO), and $\text{NO}_3^- + \text{O}$ supplemented to different forage:concentrate ratios on rumen fermentation properties.

Forage: Concentrate	Feed additive	Oils	CH ₄	NH ₃	VFA				AA:PA	PP	pH
					AA	PA	BA	TVFA			
40:60	Control	Oil-free	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	Oil-free	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}
60:40	Control	Oil-free	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	Oil-free	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
Forage: Concentrate (F:C)											
40:60			12.44	8.23	46.86	19.14	15.50	86.43	2.46	32.16	6.01
60:40			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
NaNO ₃			8.62	7.25	46.52	19.09	14.71	85.15	2.46	29.97	6.04
Oils											
Oils-free			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											
F:C			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
FA			<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
F:C × FA			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.031	0.013

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 ($\times 10^4$ ml), NO_3^- : nitrate, HO: hazelnut oil, SO: soybean oil, S.E.M: Standard error of means

Forage: Concentrate	Feed additive	Oils	CH ₄	NH ₃	VFA				AA:PA	PP	pH
					AA	PA	BA	TVFA			
F:C × Oils			<0.001	0.008	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.033
FA × Oils			0.015	0.004	<0.001	<0.001	<0.001	<0.001	<0.001	0.004	0.008
F:C × FA × 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											

3.2. NH₃ concentration in the rumen fluid.

After 48 hours of incubation, F:C ratio ($p<0.001$), FA ($p<0.001$), O ($p<0.001$), F:C x FA ($p<0.05$), F:C ratio x O ($p<0.05$), FA x O ($p<0.05$) and F:C ratio x FA x O ($p<0.05$) affected NH₃ concentration. In the present study, it was found that NH₃ concentration ($p<0.001$) increased as concentrate level increased. But, in both forage:concentrate ratios, NO₃⁻ and NO₃⁻ + oil treatment groups led to the lowest concentration of ammonia. Especially NO₃⁻ + SO treatment group was found to have lowest ammonia concentration. By the way, in both forage:concentrate ratios urea + oils treatments (control group) had higher NH₃ 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 F:C ratio, FA, O, and F:C ratio x FA, F:C ratio x O, FA x O, F:C ratio x FA x O interactions (Table 3). With the high concentrate level in rations (40F:60C), PA, BA, TVFA concentrations were high, whereas with the high forage content (60F:40C), AA, AA:PA ratio and pH were high. By the way, in 40F:60C and 60F:40C, **while NO₃⁻, NO₃⁻ + oils increased propionic acid and butyric acid concentration, they decreased acetic acid concentration, TVFA, AA:PA ratio, and pH.** In the presence of the high concentrate, NO₃⁻ and NO₃⁻ + O supplementation increased PA and BA concentrations ($p<0.001$), but decreased AA, TVFA production, AA:PA ratio and pH ($p<0.001$).

3.4. Protozoa population

Protozoa number was affected by F:C ratio ($p<0.001$), FA ($p<0.001$), O ($p<0.001$), F:C ratio x FA ($p<0.05$), F:C ratio x O ($p<0.001$), FA x O ($p<0.05$) and F:C ratio x FA x O interaction ($p<0.05$). Protozoa number was found to be high in the high concentrate level (40F:60C) compared to 60F:40C which is high in forage level. At the end of 48 hours of fermentation, in both forage:concentrate ratios NO₃⁻ and a combined effect of NO₃⁻ + O supplementation decreased protozoa population ($p<0.05$). In both forage:concentrate ratios, the mitigation effect of NO₃⁻ + SO supplementation on protozoa number was more evident.

4. Discussion

4.1. CH₄ production

A low CH₄ production recorded in high forage level (60F:40C) may be associated with the presence of high levels of secondary metabolites (saponin and condensed tannin) in alfalfa plant used as a roughage source (Kozłowska et al., 2020). Previously, Castro-Montoya et al. (2012) found that rations using quillaja plant with high saponin content as a roughage source decreased CH₄ production compared to rations with high concentrate feed. Likewise, in some studies, saponin has been found to reduce CH₄ production (Morgavi et al., 2012; Jayanegara et al., 2014; Chen et al., 2019). While some researchers found that a low condensed tannin (CT<0.001%) content in alfalfa hay decreased CH₄ production, some researchers reported a decrease in CH₄ production due to the low NDF and a high CP 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 NO₃⁻ supplementation on CH₄ production in both forage:concentrate ratios was different. In this study the increase of forage level (60F:40C) led to a decrease of CH₄ production. An interaction was found between ration type (roughage/concentrate ratio) and CH₄ reducing agents (such as nitrate) in cattle (Alvarez-Hess et al., 2019). A high CH₄ production found

in a high concentrate level, is consistent with some studies (Hristov et al., 2015; 2017; Moate et al., 2017; 2019). In this study, a relationship between CH₄ 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 NO₃⁻ + O added rations decreased CH₄ production in both forage:concentrate ratios (p<0.001). Some researchers reported that a combined effect of oils (rich MUFA or PUFA) and NO₃⁻ is an effective method to reduce CH₄ production in rumen (Leng and Preston, 2010; Yang et al., 2016). It has been determined that a lower effect of NO₃⁻ on CH₄ production is associated to NO₃⁻ 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 CH₄ production (El-Zaiat et al., 2014; Olijhoek et al., 2016). Reduction of nitrate to nitrite and then to NH₃ reduces H ions concentration required for the conversion of the CO₂ to CH₄ compound in the rumen and thus CH₄ production decreases (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, the reducing effect of NO₃⁻ on CH₄ is more evident in rations with a high forage which is rich in condensed tannins or saponins (Pal et al., 2014). This is consistent with our results. In our study, while a high CH₄ production was observed in 40F:60C (12.44 ml), a lower CH₄ production (9.09 ml) was recorded in low a high forage level (Table 3). This result is related to the increase in saponin and condensed tannins level and their effects on CH₄ production in a high forage level.

In our study, oils used were rich in PUFA (SO) and MUFA (HO). As expected, SO with a high content of PUFA decreased CH₄ production at a higher level than HO. This finding is consistent with studies reporting that the mitigation effect of fats on CH₄ production is related to degree of unsaturation (Rodrigues et al., 2017; Vargas et al., 2017).

In both forage:concentrate ratios the higher negative effect of SO (rich in PUFA) in the reduction of CH₄ 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 CH₄ 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 CH₄ mitigation was found (Dong et al., 1997). In both forage:concentrate ratios HO decreased CH₄ production. Likewise, in some studies canola oil (22% linoleic acid, 11% linolenic acid, and 54% oleic acid) caused a reduction of CH₄ 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 CH₄ production (Freitas et al., 2018; Nur Atikah et al., 2018).

In present study, the decrease in CH₄ production due to O addition can be associated with the decrease in protozoa population. In both forage:concentrate ratios, it was determined that NO₃⁻ + O supplementation caused a higher decrease in CH₄ production compared to the use of O and NO₃⁻ 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 CH₄ production decreases when nitrate and flaxseed oil (high in MUFA) are added to ration.

4.2. NH₃ concentration

In the current study, rumen NH₃ values determined for rations used in both forage:concentrate ratios are above the recommended minimum NH₃ concentration (4.39 to 7.32 mmol/l) (Satter and Slyter, 1974), which is considered sufficient for maximum microbial growth rates. The high NH₃ concentration found in the high concentrate level (40F:60C) might be related to the high number of proteolytic bacteria in rumen. Because, proteolytic bacteria increase ruminal NH₃ 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 forage level (60F:40C) decreased NH₃ 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 NH₃ production. Our results were consistent with some previous studies (Belanche et al., 2016; Jadhav et al., 2018).

In our study, the forage:concentrate ratios associated with NO₃⁻ supplementation decreased NH₃ (p<0.001). NO₃⁻ is converted to nitrite, which has a toxic effect on rumen bacteria, and therefore NO₃⁻ addition reduces NH₃ 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 .

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 forage:concentrate ratios (40F:60C, and 60F: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 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 forage:concentrate ratios 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 the both forage:concentrate ratios. 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 forage/concentrate ratio. In this study while a high concentrate level decreased pH, a high forage level increased pH. Although, it was found that NO_3^- addition link to forage:concentrate 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. A decreased in pH due to the high concentrate level can be associated to a high starch content which creates an environment to inhibit nitrate and nitrite metabolism. This means that a high concentrate level 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 level (Table 3)

In the present study, oil addition associated to forage:concentrate 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 forage:concentrate 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 F:C ratios, FA, O, F:C ratios x FA, F:C ratios x O, FA 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 level 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 forage level, 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 CH₄ 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 NO₃⁻ 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 NH₃ concentration, AA and AA: PA ratio decreased in the rumen. But, a decrease in BA (due to NO₃⁻ supplementation) was caused by the rapid reduction of NO₃⁻ (to nitrite then ammonia) which use up the electrons needed for the production of BA. However, a high forage level decreased BA concentration. This was due to the combined effect of tannin and NO₃⁻. 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 NO₃⁻ supplementation can be explained by the use of free hydrogen for production of NH₃ 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 NO₃⁻ 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 NO₃⁻ + O on VFA and AA: PA ratio changed according to the source of fatty acids (MUFA and PUFA). For that, NO₃⁻ + SO (rich in PUFA) associated with forage: ratios decreased AA concentration but it increased PA and BA concentrations. This result can be explained by the simultaneous effect of NO₃⁻ (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 NO₃⁻ + SO on AA, CH₄, NH₃, TVFA and AA:PA ratio. However, while NO₃⁻ 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, CH₄, NH₃, 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 CH₄ were more decreased due to the combined effect of NO₃⁻ + SO which can be explained by biohydrogenation of PUFA and NO₃⁻ mechanism (transformation of NO₃⁻ to nitrite then to NH₃) for obtaining PA having different and associative mechanism for using available hydrogen. Use of NO₃⁻ 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 forage level (p<0.001). Previously, it was shown that a high concentrate level 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 forage (rich in secondary metabolites: saponins and tannins) content of ration link to NO₃⁻ addition caused a decrease in the protozoa number. This can be explained by the combine effect of NO₃⁻ and saponins which acted negatively on protozoa population. Our findings are consistent with those of Lin et al. (2013).

In the present study, NO₃⁻ added rations associated with forage:concentrate ratios decreased PP. Otherwise, nitrite which come from a transformation of nitrate, inhibits rumen protozoa population and thus CH₄ production. This is consistent with findings of Iwamoto et al. (2001).

Furthermore, in our study, there is a parallelism between NH₃ concentration and PP in a high forage level, and this finding was reported by some studies (Hu et al., 2005; Liu et al., 2018).

In our study, use of NO₃⁻ 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 NO₃⁻ + SO decreased protozoa population more than individual use of NO₃⁻ and O. This can be explained by the simultaneous mitigation effect of NO₃⁻ 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 forage level (because of a presence of saponins and condensed tannins in high level) decreased CH_4 , AA, NH_3 , TVFA, PP, and AA:PA ratio. While the 60F: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 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 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 advantageous to reduce methane production and protozoa population without a negative effect on PA and BA concentrations.

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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Conflict 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. GARİPOĞLU 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. GARİPOĞLU. All authors read and approved the final manuscript.

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