The Effect of Natural Feed Additives on Methane Emissions, Nutrient Intake, Digestibility and Rumen Fermentation Parameters

2016

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天然素材からなる飼料添加物の反芻家畜への給与が，消化管からのメタン産生，養分摂取，飼料消化率および第一胃内発酵に及ぼす影響

2016

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博士後期課程　畜産衛生学専攻

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General Introduction

The contribution of livestock production towards environmental pollution is becoming of great concern because of the emissions of greenhouse gases (GHG), such as carbondioxide (CO_2), methane (CH_4) and ammonia-N (NH_3-N). In addition, the production of CH_4 during the enteric fermentation of feeds in the rumen is correlated with the loss of GE from the consumed feed (Szumacher-Stabel and Cieslak, 2012). While ruminants play an important role as an essential source of high-quality protein in human diets, they are also a major source of GHGs. According to FAO estimates (Opio et al., 2013), the greatest source of CH_4 in ruminant production is enteric fermentation, which accounts for approximately 47% of the sector's GHG emissions and more than 90% of total CH_4 emissions. As a GHG, CH_4 is 25 times stronger than CO_2 (Opio et al., 2013), and its effect will become more pronounced in the short term because ruminant production is increasing worldwide to meet an ever-increasing demand for milk and meat (Becker et al., 2013). Thus, identifying alternative solutions to this major constraint is a concern of both environmental protection and nutrient utilization.

There is a growing interest in exploiting natural feed additives, aimed for use in animal nutrition and livestock production that offer potential to improve rumen fermentation efficiency while reducing GHG emissions. Several strategies have been explored to mitigate CH_4 production using feed additives in ruminants without any adverse effect on nutrient intake, digestibility and efficiency of utilization. Among the major natural feed

additives, Sunphenon 30S-O and Euglena were considered for this study based on their potential as a source of catechins (precursor of condensed tannin) and fatty acids respectively which could help to mitigating CH_4 emissions and improve efficiency of nutrient utilization. The presence of condensed tannins (CT) and dietary lipids or their constituent alone, reduce CH_4 emissions but at high intakes they can reduce digestibility and dry matter (DM) intake.

Tea is one of the most popular beverages in the world (Khokhar and Magnusdottir, 2002); annual production totals approximately 4 million tons (Bordoloi, 2012). As part of the production of ready-made tea drinks packaged in bottles, packs and cans, beverage companies discard a large amount of tea grounds annually (Wang and Xu, 2013). Green tea extracts contain polyphenolic compounds that account for 30% of the dry weight of leaves (Mukhtar and Ahmad, 2000), and *in vivo* and *in vitro* studies (Mitsumoto et al., 2005; Wang and Xu, 2013; Zhong et al., 2009) have indicated that green tea polyphenols improve growth performance, meat quality and shelf life due to their antioxidant properties in cattle, sheep and goats. Flavanols, generally known as catechins, are the most abundant polyphenols in green tea leaves and account for nearly 80-90% of the total polyphenol content (Htay et al., 2008; Riemersma et al., 2001). The physiological effects of green tea depend on a variety of catechins, including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), all of which are usually present in high concentrations in tea leaves (Spencer, 2003). The structural formation of EGCG (also known as condensed tannin) is believed to be responsible for the pronounced physiological activity of tea, including its antioxidant effects (Htay et al., 2008). Sunphenon 30S-O is obtained from green tea (*Camellia sinensis*) leaves, and it is standardized for its catechin content. Catechin is the only polyphenol present in Sunphenon 30S-O, and the inclusion of such catechin-containing natural plant extracts in ruminant rations might influence CH_4 emissions, nutrient intake, digestibility and other rumen fermentation parameters.

Algae represent one of the most efficient converters of solar energy to biomass (Masojikek, and Prasil, 2010). The use of algae has a great potential not only in the pharmaceutical and food industries (Lee, 2001), but also as an additive to livestock feed (Rasoul-Amini et al., 2009). Such supplementation of ruminants is an effective method for increasing concentrations of poly unsaturated fatty acids in the ruminant's product. Changes in the fatty acid profile probably related to changes in the population of rumen bacterial flora (Toral et al., 2012). Microalgae are one of the most promising biological resources, as these organisms are rich sources of vitamins, minerals, proteins, polyunsaturated fatty acids, antioxidants, etc. (Pulz and Gross, 2004) and can be used to enhance the nutritional value of animal feed, reflecting the well-balanced chemical composition of these microphytes. The inclusion of microalgal biomass in small quantity positively affects the physiology of animals, as antibacterial action, improve gut function, feed conversion and reproductive performance have been reported (Harel and Clayton, 2004). A number of nutritional studies have demonstrated the suitability of microalgae biomass as a potential substitute for conventional protein supplements, such as soybean and fish meal (Dajana et al., 2013).

Carbon dioxide fixation through *Euglena gracilis* is effective and economical (Chae et al., 2006), thereby lowering the greenhouse effect and climate changes through the absorption of increasing CO_2 emissions in the atmosphere. Microalgae can be cultivated in areas unsuitable for other plants with several fold higher production and can effectively utilize and remove pollutants (e.g., nitrogen and phosphorus) from water (Gouveia et al., 2008). Thus Euglena, due to its rich source of fatty acid, protein and other biologically active compounds, inclusion of these micro algae in the ration of ruminants may influence the emissions of CH_4, rumen fermentation and efficiency of nutrient utilization.

Strategies to reduce CH_4 emission and improve efficiency of nutrient utilization can be categorized in to the following main groups: manipulating the animals' diet; manure

management; breed improvement; improved forage production and feed treatment and silage making. In our study emphasis was given to manipulation of the ruminant diet by the use of natural feed additives such as Sunphenon 30S-O and Euglena. The hypothesis was that inclusion of natural feed additives (Sunphenon 30S-O and Euglena) might influence the rumen fermentation activity, intake, digestibility and CH_4 emission.

To the best of our knowledge, there is no information available on the effect of Sunphenon 30S-O and Euglena on rumen fermentation and intake. Thus this experiment was conducted to investigate the influence of Sunphenon 30S-O (containing a standardized level of catechin, 205 g/kg DM) and Euglena on nutrient intake, digestibility, CH_4 emissions, VFA concentrations, NH_3-N concentrations, the protozoa population and rumen degradability.

With this background the objectives of this study were

- To investigate the effects of Sunphenon 30S-O (standardized green tea extract) on *in vitro* CH_4 emission, rumen degradability and rumen fermentation
- To evaluate the effects of Sunphenon 30S-O (standardized green tea extract) on *in vivo* nutrient intake, nutrient digestibility, nutrient balance and CH_4 emission by sheep
- To investigate the effects of Euglena *(Euglena gracilis)* supplemented to diet (forage: concentrate ratios of 60:40) on the basic ruminal fermentation and CH_4 emissions in *in vitro* condition
- To evaluate the effects of Euglena *(Euglena gracilis)* on intake, digestibility and rumen fermentation parameters.

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Chapter One

The effects of Sunphenon 30S-O (standardized green tea extract) on *in vitro* methane production, rumen fermentation, and nutrient degradability

Abstract

Different concentrations of Sunphenon 30S-O (standardized green tea extract) were investigated for their effects on *in vitro* methane (CH_4) production, volatile fatty acid (VFA) concentration, ammonia-N (NH_3-N) concentration, protozoa population, pH, oxidation reduction potential (ORP) and nutrient degradability. The treatments considered were Sunphenon 30S-O at concentrations of 0.0, 20, 40, and 50 g/kg dry matter (DM) of Guinea grass (*Panicum maximum*) hay. Treatments with buffered rumen fluid were incubated for 24 h using *in vitro* continuous gas production and *in vitro* digestion techniques. The data were subjected to polynomial regression analysis. Methane production (ml 24 h^{-1}) reduced linearly ($P<0.001$) while carbon dioxide (CO_2) production (ml 24 h^{-1}) reduced linearly ($p<0.001$) and quadratically ($p<0.006$) with increasing concentrations of Sunphenon 30S-O. Total VFA concentration (mmol/L) and NH_3-N production (mg/ml) reduced at an increasing rate (linear $P<0.001$; quadratic $P<0.004$) with increasing concentrations of Sunphenon 30S-O. The total protozoa population also declined at an increasing rate linearly and quadratically ($P<0.001$), with increasing concentrations of Sunphenon 30S-O. There was a linear ($p<0.003$) and quadratic ($p<0.024$) reduction in the acetate to propionate ratio at 50 g/kg DM inclusion, which did not show any significant effects on ORP ($P>0.05$) and pH ($P>0.05$). *In vitro* dry matter degradability (IVDMD) reduced linearly and quadratically ($P<0.001$) with increasing concentration of Sunphenon 30S-O. *In vitro* organic matter degradability (IVOMD) also followed the same trend and reduced linearly ($P<0.001$) and quadratically ($p<0.004$) with increasing concentration of

Sunphenon 30S-O. Similarly *in vitro* ruminal crude protein degradability (IVRCPD) reduced linearly ($p<0.001$) and tended to reduce quadratically ($P=0.056$). The finding of this study suggests that addition of Sunphenon 30S-O reduced CH_4 emission in a dose dependent manner. However, when the inclusion of Sunphenon goes beyond 40 g/kg DM of the ration, reduction in CH_4 emission was associated with losses in OM degradability, total protozoa population and total VFA concentration. Thus the findings of this *in vitro* study suggests that optimum reduction of CH_4 (9.5%), without any significant effect on other ruminal fermentation parameters can be obtained at lower to medium concentrations of Sunphenon 30S-O

1. Introduction

Climate change is one of the greatest obstacles facing the world today, and its association with the emission of greenhouse gases (GHGs), such as CO_2 and CH_4, is well known. While ruminants play an important role as an essential source of high-quality protein in human diets, they are also a major source of GHGs. According to FAO estimates (Opio et al., 2013), the greatest source of CH_4 in ruminant production is enteric fermentation, which accounts for approximately 47% of the sector's GHG emissions and more than 90% of total CH_4 emissions. As a GHG, CH_4 is 25 times stronger than CO_2 (Opio et al., 2013), and its effect will become more pronounced in the short term because ruminant production is increasing worldwide to meet an ever-increasing demand for milk and meat (Becker et al., 2013). Therefore, reducing CH_4 emissions from ruminant livestock will play a significant role in decreasing environmental pollution, provided that nutrient utilization efficiency is not affected.

Modifying the composition of animal diets is often regarded as an option to minimize ruminal CH_4 emissions (Becker et al., 2013), and condensed tannin-containing legume forages (Animut et al., 2008 with 50-151 g CT/kg DM; Min et al., 2002 with 32 g CT/kg DM; Tavendale et al., 2005 with 91-107 g CT/kg DM; Williams et al., 2011 with 5-49 g CT/kg DM; Woodward et al., 2001 with 26 g CT/kg DM) and tannin extracts (Beauchemin et al., 2007 with 18 g CT/kg DM; Carulla et al., 2005 with 25 g/kg DM; Hess et al., 2006 with 25 g CT/kg DM; Pellikaan et al., 2011 with 100 g CT/kg DM; Tan et al., 2011 with 20-60 g CT/kg DM) have been extensively investigated for their ability to inhibit ruminal CH_4 production. Tannins reduce CH_4 emissions by suppressing protozoa and other hydrogen-producing microbes thus interfering with methanogenesis (Patra, 2010; Tavendale et al., 2005).

Tea is one of the most popular beverages in the world (Khokhar and Magnusdottir,

2002); annual production totals approximately 4 million tons (Bordoloi, 2012). As part of the production of ready-made tea drinks packaged in bottles, packs and cans, beverage companies discard a large amount of tea grounds annually (Wang and Xu, 2013). Green tea extracts contain polyphenolic compounds that account for 30% of the dry weight of leaves (Mukhtar and Ahmad, 2000), and *in vivo* and *in vitro* studies (Mitsumoto et al., 2005; Wang and Xu, 2013; Zhong et al., 2009) have indicated that green tea polyphenols improve growth performance, meat quality and shelf life due to their antioxidant properties in cattle, sheep and goats. Flavanols, generally known as catechins, are the most abundant polyphenols in green tea leaves and account for nearly 80-90% of the total polyphenol content (Htay et al., 2008; Riemersma et al., 2001). The physiological effects of green tea depend on a variety of catechins, including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), all of which are usually present in high concentrations in tea leaves (Spencer, 2003). The structural formation of EGCG (also known as condensed tannin) is believed to be responsible for the pronounced physiological activity of tea, including its antioxidant effects (Htay et al., 2008). Sunphenon 30S-O is obtained from green tea (*Camellia sinensis*) leaves, and it is standardized for its catechin content. Catechin is the only polyphenol present in Sunphenon 30S-O, and the inclusion of such catechin-containing natural plant extracts in ruminant rations might influence CH_4 emissions, nutrient intake, digestibility and other rumen fermentation parameters.

To the best of our knowledge, there is no information available on the effect of Sunphenon 30S-O on rumen fermentation, thus this experiment was conducted to investigate the influence of Sunphenon 30S-O (containing a standardized level of catechin, 205 g/kg DM) on nutrient intake, digestibility, CH_4 emissions, VFA concentrations, NH_3-N concentrations, the protozoa population and rumen degradability.

2. Materials and Methods

2.1. Sunphenon 30S-O.

Sunphenon 30S-O, which is standardized for catechin content (205 g/kg DM), was obtained from the leaves of traceable green tea (*Camellia sinensis*) via extraction by water infusion and decaffeination using approved food-grade solvents. Catechin is the only polyphenol present in Sunphenon 30S-O, which contains water soluble fibers as filler and whose chemical composition and major catechin components are presented in Table 1. Samples of Sunphenon 30S-O were purchased from Taiyo Kagaku Co., Ltd., Japan; Sunphenon® extracts are food grade and approved by the Japanese Foundation for Health and Nutrition for specific medical uses. They are certified organic and possess an excellent tea taste and maintain good stability in beverages.

2.2. Rumen fluid sampling

Two ruminally fistulated, non-lactating Holstein cows (600 kg average BW) were used as rumen fluid donors. The cows were maintained on a daily diet of 10 kg orchard grass hay (OM, 980 g/kg; CP, 132 g/kg; NDF, 701 g/kg; ADF, 354 g/kg; lignin, 40 g/kg; and GE, 18.02 MJ/kg; DM basis) with free access to clean drinking water and mineral blocks (Fe, 1836 mg; Cu, 377 mg; Co, 66 mg; Mg, 1046 mg; Zn, 1235 mg; I, 77 mg; Se, 33 mg; vit E, 5000 mg; Na, 962 g/1 kg). Rumen liquor was collected from the two cows just before feeding (0 h) using a vacuum line and strained through a woven nylon cloth into a thermos flask that had been pre-heated (39ºC) with hot water. All animal management and sampling procedures were approved by the Obihiro University of Agriculture and Veterinary Medicine Animal Care and Use Committee.

2.3. Experimental treatments for in vitro fermentation

The experimental samples were oven-dried at 60°C for 48 h and stored in sealed

containers under dry, cool conditions prior to use. Four treatments were prepared that consisted of different concentrations of Sunphenon 30S-O and Guinea grass (*Panicum maximum*) hay as follows: 10 g of Guinea grass hay (Control, T1); 9.8 g of Guinea grass hay + 0.2 g of Sunphenon 30S-O (T2); 9.6 g of Guinea grass hay + 0.4 g of Sunphenon 30S-O (T3) and 9.5 g of Guinea grass hay + 0.5 g of Sunphenon 30S-O (T4). The effects of each treatment on CH_4 emissions, VFA concentrations, NH_3-N concentrations, pH, oxidation reduction potential (ORP) and the protozoa populations were tested *in vitro* for 24 h at 39°C using a continuous gas quantification system, as previously described by Sar et al. (2005).

Table 1. Chemical composition of experimental feeds

Item [a]	Guinea grass hay	Concentrate mixture	Sunphenon 30S-O
DM (g/kg)	956	958	944
OM (g/kg DM)	909	918	913
Ash(g/kg DM)	84.4	76.5	85.6
CP (g/kg DM)	147	165	137
EE (g/kg DM)	16.0	33.6	6.20
GE (MJ/kg DM)	17.1	16.7	15.9
NDF (g/kg DM)	609	232	20.0
ADF (g/kg DM)	303	78.3	9.00
ADL(g/kg DM)	36.2	35.6	2.00
(+)-catechin (g/kg DM)	-	-	3.00
EC (g/kg DM)	-	-	21.0
EGC (g/kg DM)	-	-	79.0
EGCG (g/kg DM)	-	-	84.0
ECG (g/kg DM)	-	-	18.0

[a]DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; GE: gross energy; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL acid detergent lignin; EC epi catechin; EGC epi galo catechin; EGCG epi galo catechin galate; ECG epi catechin galate

Briefly, samples of rumen fluid were obtained from two non-lactating Holstein cows and strained and combined in equal volumes. The buffer was prepared according to McDougall (1948), sterilized by autoclaving and flushed with CO_2 for 1 h prior to being dispensed into fermentation vessels. Fermentation was allowed to continue for 24 h at 39°C, and rumen fluid was added to the buffer in a ratio of 1:4. The source of replication (n=4) in the experimental model was provided by rumen fluid inocula collected on separate occasions, and the treatments were randomly assigned to incubation vessels for each incubation period. The gas output from each fermentation vessel was measured for 10 minutes at 30-min intervals. Samples of the incubation medium were collected after 24 h of incubation and were stored at -20°C for the analysis of NH_3-N and VFA, and at the end of each 24-h incubation period, all incubations were stopped, the contents were discharged, and the fermenters were thoroughly washed and autoclaved. The fermenters were then re-charged with fresh buffer and inoculum to begin the next 24-h incubation period.

2.4. Analysis of methane, carbon dioxide and volatile fatty acids

Methane production from each fermentation vessel was measured continuously with automatic infrared CH_4 (EXA IR, Yokogawa Electric Corporation, Tokyo, Japan) and CO_2 (Model RI-555, Riken Keiki Co. Ltd, Tokyo, Japan) analyzers installed in the *in vitro* continuous gas quantification system (Takasugi Seisakusho Co. Ltd, Tokyo, Japan). Total VFA and its components were determined with a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (ULBON HR-52, 0.53 mm ID × 30 m, 3.0 μm) using 2-ethyl-n-butyric acid as an internal standard; samples were prepared for analysis according to Sar et al. (2005). The pH and ORP of the fermentation media were monitored in each vessel at 1-min intervals (HP-21P, Toa electronics Ltd., Tokyo, Japan). All data were pooled and stored on a computer via an interface with the analyzers.

2.5. In vitro nutrient degradability

In vitro nutrient degradability was estimated by following the first stage of the digestion technique described by Tilley and Terry (1963). Triplicate 0.5 g samples of Guinea grass hay (control, T1), 0.49 g of Guinea grass hay + 0.01 g Sunphenon 30S-O (T2), 0.48 g of Guinea grass hay + 0.02 g of Sunphenon 30S-O (T3) and 0.475 g of Guinea grass hay + 0.025 g of Sunphenon 30S-O (T4) were weighed and placed into a 100-ml plastic bottles, and 40 ml of McDougall's buffer (McDougall, 1948) was added to each bottle and pre-warmed to 39°C. Then, 10 ml of strained rumen fluid was dispensed into each bottle and sealed under a continuous supply of CO_2 gas. The mixture was incubated at 39 ºC for 24 h and carefully shaken occasionally. After incubation termination, the contents were filtered through pre-weighed Gooch crucibles; the amount of residual DM was determined, and the loss in weight was considered the IVDMD. This was followed by ashing of the residues for the estimation of *in vitro* organic matter degradability (IVOMD), and *in vitro* rumen crude protein degradability (IVRCPD) was estimated at the end of the incubation period by filtering the contents through laboratory-grade filter paper (Grade 1, 100 circles/125 mm, Toyo Roshi, Ltd, Japan). The amount of nitrogen in the residues was analyzed by the Kjeldahl method (AOAC 984.13), and the disappearance was calculated from the differences in the protein content of the sample before and after incubation. *In vitro* rumen degradability experiments were repeated four times.

2.6. Chemical analysis

Experimental samples were analysed for DM by drying at 135 °C for 2 h (930.15), and OM, total ash (942.05) and ether extract (EE) (920.39) were determined according to the procedures of AOAC (1995). N was determined by the Kjeldahl method (984.13) (AOAC, 1995) using an electrical heating digester (FOSS Tecator^{TM} Digester, Tokyo, Japan) and an automatic distillation apparatus (FOSS Kjeltec^{TM} 2100, Tokyo, Japan), and

crude protein (CP) was then calculated as the amount of N × 6.25. Neutral detergent fibre (NDF) was estimated without amylase and expressed inclusive of residual ash according to the method described by Van Soest et al. (1991), which was also used to determine acid detergent fibre (ADF) and lignin. The ADF was expressed inclusive of ash, and lignin was determined by the solubilization of cellulose with sulphuric acid. The gross energy (GE) content of the samples was analysed in a Shimadzu auto-calculating bomb calorimeter (CA-4AJ, Shimadzu Corporation, Japan), and the NH_3-N concentration was analysed according to Conway and O'Malley (1942).

The components of the total catechins in Sunphenon 30S-O were analysed by Japan Food Research Laboratories using HPLC (Shimadzu LC-MS with an LC-20AD column and a SPD-20A detector). EC, EGC, EGCG, and ECG were separated by a reverse phase mechanism on a C18 column containing water, methanol and 0.02-mol/L phosphate buffer (pH=3.0) as the mobile phase gradient. EC was detected and quantified by fluorescence with excitation at 280 nm and measured at 315 nm with a flow rate of 1.0 ml/min. EGC, EGCG and ECG were detected by ultraviolet light at 270 nm with a 1 ml/min flow rate, and mass spectra were collected by Shimadzu LC-MS and electrospray ionization mass spectrometry ES/MS). Catechin was separated on an Atlantis T3 2.1-mm*150-mm column with acetonitrile, acetic acid and water linear gradient ionization.

2.7. Statistical analysis

The effects of Sunphenon 30S-O on *in vitro* gas production, nutrient degradability and rumen fermentation parameters were tested by polynomial regression analysis, using SAS (2010) statistical software version 9.3. *In vitro* rumen degradability was completed in four runs with each sample incubated in triplicate. The average of replication within a run was considered to be a statistical unit. In cases of *in vitro* gas production, each treatment was incubated four times on different days (statistical replicates) The total effects included in the model for each variable were four replications and four treatments. Linear, quadratic

and cubic contrasts of the treatment means were assessed. Differences among the means were identified using Tukey's multiple comparisons. Effects were considered significant when $P<0.05$, and trends were discussed at $0.05 <P<0.10$.

3. Results

3.1. Chemical composition of the experimental feeds

The total catechin content of Sunphenon 30S-O in this study was 20.5 g/100 g. Components of the catechins contained in Sunphenon 30S-O are indicated in Table 1. EGCG and EGC constitute 80% of the total catechins in Sunphenon 30S-O. Sunphenon 30S-O contained comparable amounts of total CP, OM, ash and GE compared to the Guinea grass hay used as a substrate (Table 1). Sunphenon 30S-O contains relatively lower amount of fatty acid and its fiber content (NDF and ADF) is negligible.

3.2. In vitro *methane emissions and rumen degradability*

The effects of Sunphenon 30S-O at concentrations of 0.0, 20, 40 and 50 g/kg DM of the substrate on the nutrient degradability, CH_4 and CO_2 emissions are indicated in Table 2. Methane production (ml 24 h^{-1}) was reduced linearly ($P<0.01$) with increasing concentrations of Sunphenon 30S-O (Fig 1), and CO_2 production (ml 24 h^{-1}) followed the same trend, declining linearly ($P<0.01$) and quadratically ($P<0.01$). *In vitro* rumen DM degradability was reduced linearly and quadratically ($P<0.01$) at an increasing rate with higher concentrations of Sunphenon 30S-O. A similar linear ($P<0.01$) and quadratic ($P<0.01$) trend was observed for IVOMD, and IVRCPD was reduced linearly ($P<0.01$) but tended to decrease quadratically ($P=0.06$) Fig. 2.

3.3. The effects of Sunphenon 30S-O on in vitro rumen fermentation

The total concentrations of VFA (mmol/L) and NH_3-N (mg/ml) decreased at an increasing rate (linear $P<0.01$; quadratic $P<0.01$) with increasing concentrations of Sunphenon 30S-O (Fig. 3). The acetate-to-propionate ratio decreased linearly ($P<0.01$) and quadratically ($P<0.05$). The molar proportions of acetate were not affected ($P>0.05$), whereas the proportion of propionate increased linearly ($P<0.01$) and quadratically ($P<0.05$). Valeric acid tended to decrease linearly ($P<0.10$), and the molar proportion of butyrate declined (linear $P<0.01$; quadratic $P<0.01$) with increasing concentrations of Sunphenon 30S-O. The protozoa population also declined linearly and quadratically ($P<0.01$) at an increasing rate with increasing concentrations of Sunphenon 30S-O (Table 3). The addition of Sunphenon 30S-O did not have any significant effects on ORP ($P>0.05$) and pH ($P>0.05$).

Table 2. The effect of Sunphenon 30S-O on *in vitro* CH_4 emission, CO_2 production and nutrient degradability

Item[1]	Level of Sunphenon 30S-O (g/kg DM)				SEM	Contrasts[2]		
	0.0	20	40	50		L	Q	C
CH_4(ml24h^{-1})	36.0^{a}	36.4^{a}	32.6^{b}	30.8^{b}	0.398	<0.001	0.241	0.113
CH_4(ml/g digestible DM)	6.59^{a}	6.56^{a}	6.00^{b}	5.68^{c}	0.046	<0.001	0.079	0.015
CH_4(ml/g digestible OM)	8.27^{a}	8.27^{a}	7.61^{b}	7.43^{b}	0.040	<0.001	0.353	<0.001
CO_2(ml24h^{-1})	396^{a}	391^{a}	370^{b}	341^{c}	1.883	<0.001	0.006	0.581
In vitro rumen degradability(24 h)								
IVDMD	0.51^{a}	0.51^{a}	0.50^{a}	0.47^{b}	0.002	<0.001	<.001	0.469
IVOMD	0.45^{a}	0.46^{a}	0.44^{a}	0.42^{b}	0.002	<0.001	0.004	0.731
IVRCPD	0.57^{a}	0.57^{a}	0.55^{b}	0.54^{b}	0.002	<0.001	0.056	0.273

[1]CH_4: methane; IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability; IVRCPD: *in vitro* rumen crude protein degradability;

$^{a-c}$ Means within a raw with different superscripts differ($P<0.05$),

[2] L= linear, Q= quadratic, C= cubic,

Table 3. The effect of Sunphenon 30S-O on *in vitro* rumen fermentation and protozoa count after 24 hours of incubation

Item[1]	Levels of Sunphenon 30S-O (g/kg DM)				SEM	Contrasts[2]		
	0.0	20	40	50		L	Q	C
Volatile fatty acids (mol/100mol)								
Acetate (A)	70.0	70.0	70.0	70.0	0.124	0.608	0.765	0.912
Propionate (P)	21.7^{b}	21.7^{b}	21.8^{b}	22.6^{a}	0.07	0.003	0.019	0.342
Butyrate	7.44^{a}	7.42^{a}	7.19^{a}	6.28^{b}	0.07	<0.001	0.007	0.493
Valeric acid	0.93	0.90	0.90	0.90	0.006	0.096	0.445	0.677
Total VFA (mmol/L)	38.5^{a}	37.9^{a}	35.2^{b}	32.4^{c}	0.158	<0.001	0.004	0.156
A:P ratio	3.23^{a}	3.23^{a}	3.21^{a}	3.09^{b}	0.012	0.003	0.024	0.371
pH(mean)	7.07	7.02	6.99	6.98	0.013	0.195	0.579	0.897
ORP (mV)	-412	-411	-412	-413	0.850	0.619	0.847	0.502
Total Protozoa (cell/l*10^{6})	3.40^{a}	3.35^{a}	3.23^{a}	2.75^{b}	0.022	<0.001	<0.001	0.182
NH_3-N (mg/ml)	27.5^{a}	27.3^{a}	27.0^{a}	25.3^{b}	0.107	<0.001	0.004	0.272

[1]VFA: volatile fatty acid; A:P: acetate to propionate ratio; ORP: oxidation reduction potential; NH_3-N, Ammonia N, [2] L = linear, Q = quadratic, C = cubic

[a-c]Means within a raw with different superscripts differ($P<0.05$)

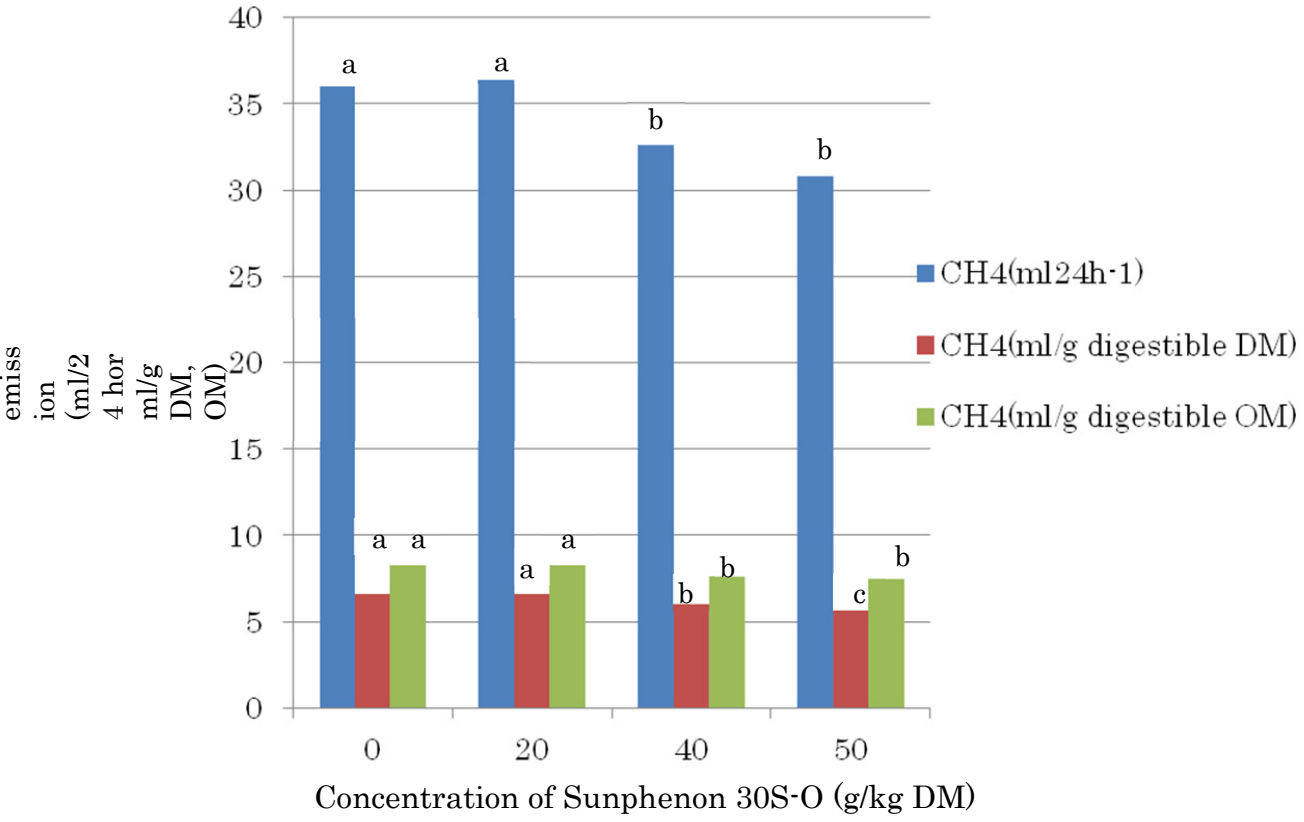

Fig1. Methane emission from *in vitro* fermentation

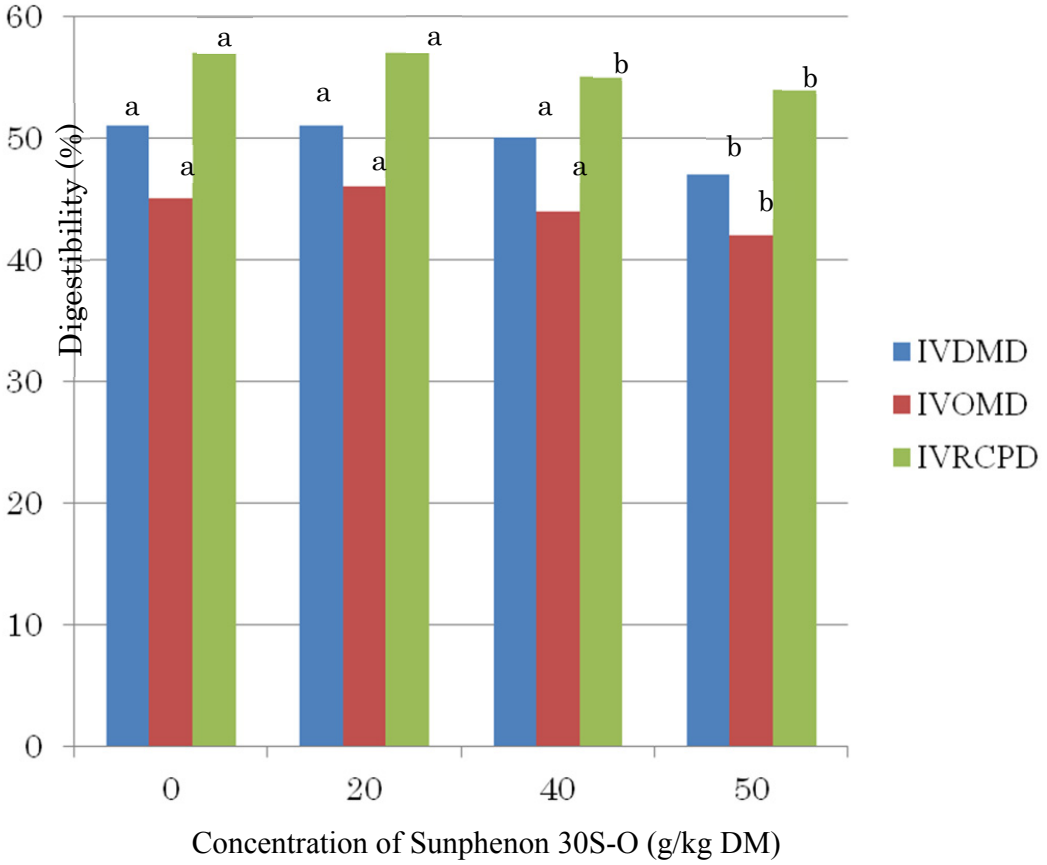

Fig2. Effect of Sunphenon 30S-O on *in vitro* dry matter, organic matter and crude protein digestibility

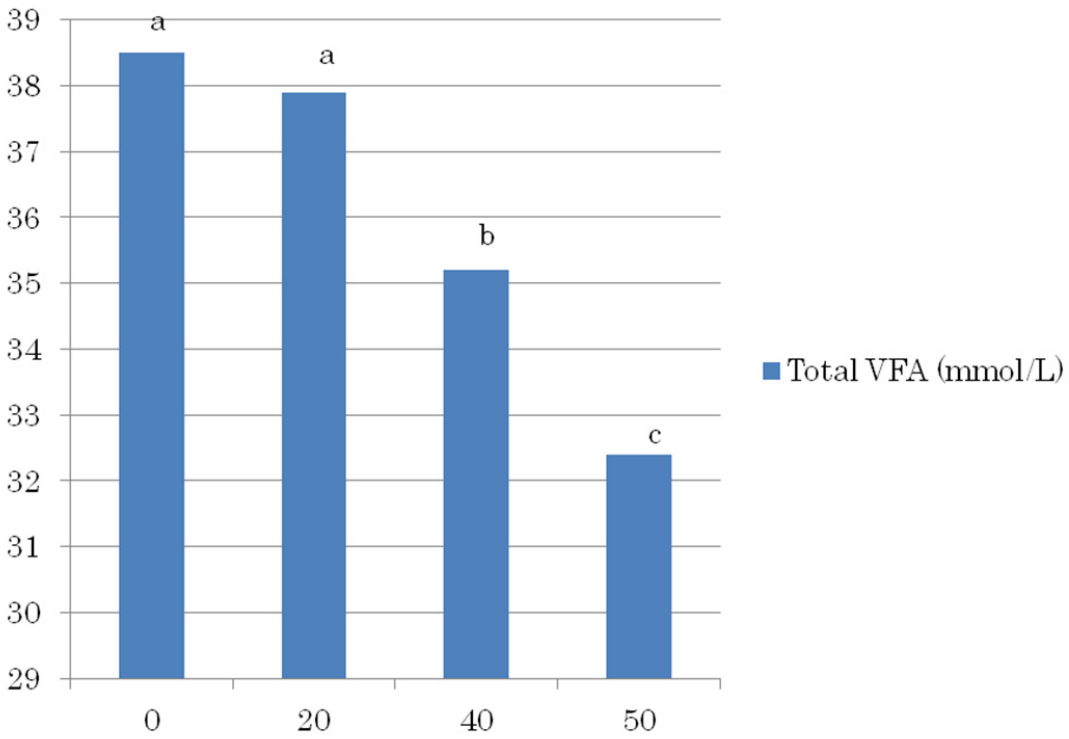

Fig3. The Effect of Sunphenon 30S-O on *in vitro* total volatile fatty acid concentration

4. Discussion

4.1. Effect on methane emissions

The inclusion of natural feed additives should be considered from the perspective of their effect on environmental safety and efficiency of nutrient utilization. The results of our findings indicate that the inclusion of Sunphenon 30S-O (4-5% of the substrate on DM basis) reduces CH_4 production in the range of 9.5 to 14.5%, while CO_2 production was reduced 6.4 to 13.8% compared to the control. In this study, 1 mol of catechin in Sunphenon 30S-O (1.0% of the substrate on DM basis) reduced the emission of 1.8 mol of CH_4 while the findings by Becker et al., (2013), suggested that catechins decreased CH_4 production in a dose-dependent manner, where 1.0 mol of catechin prevented the emission of 1.2 mole of CH_4. Bhatta et al., (2009), also reported that quebracho tannins inhibited CH_4 production by 13-45% with increasing doses (5-25%) of the substrate. In addition, Tan et al., (2011) and Patra et al., (2006a) also observed that CH_4 production decreased in the presence of condensed tannin from plant extracts. The presence of catechins (a precursor of condensed tannin) in Sunphenon 30S-O might be responsible for the reduction of CH_4 emission by affecting the activities of protozoa and associated rumen microbes. Previous work by Tavendale et al., (2005), confirmed that the inhibition of methanogen growth is due to the toxic effects of condensed tannin (CT) and that it is linked to reductions of produced CH_4.

4.2. Effects of Sunphenon 30S-O on the in vitro Protozoa population and NH_3-N concentration

Most plant compounds lead to lower CH_4 production from ruminant function as toxins that inhibit the growth of protozoa, fermentative bacteria, or methanogenes (Patra and Saxena, 2010). Tannin suppresses methanogenesis either directly or by reducing the

protozoa population, thereby reducing the methanogenes symbiotically associated with the protozoa population (Bhatta et al., 2009). In the present study, the protozoa population was reduced by 5-19% with increasing doses of Sunphenon 30S-O. Previous works (Makkar et al., 1995; Tan et al., 2011) indicated that condensed tannin inclusion reduced total protozoa counts significantly and are in agreement with our findings. Reduced protozoa numbers with an increasing intake of tannin-containing plant extracts have also been reported (Animut et al., 2008b; Patra et al., 2006a), and these decreases in the protozoa population would lead to less release of the products of protein breakdown (Van Soest, 1994). In the present study, the inhibitory effects of Sunphenon 30S-O on the protozoa population were more pronounced as the concentrations of Sunphenon 30S-O inclusion increased.

The incorporation of CT containing forages may substantially improve environmental sustainability by reducing nitrogen excretion (Williams et al., 2011). In the present study NH_3–N concentrations *in vitro* decreased with increasing concentrations of Sunphenon 30S-O and were 2 and 8% lower at 40 and 50 g Sunphenon /kg DM of the substrate, respectively compared to the control. A 27.8% reduction in NH_3–N concentrations was reported by Williams et al. (2010) in continuous cultures fed with condensed tannin containing forage diets. The observed decrease in rumen ammonia concentration was due to a decrease in protozoa numbers (Wina et al., 2005a; Wang et al., 2012). Similarly, the present study confirmed that the addition of Sunphenon 30S-O reduced the protozoa population and the NH_3–N concentration.

4.3. Effect on volatile fatty acid concentration

Fermented products and nutrient digestibility in the rumen are represented by VFA production (France and Dijkstra 2005). In our study, total VFA concentration was reduced by 8.6-15.9% with increasing concentrations of Sunphenon 30S-O (20-50 g/kg DM), and our data agree with Dschaak et al., 2011, who stated that CT extract supplementation (30 g/kg DM) of lactating cows fed a high-forage diet reduced the total VFA concentration by

6%. Tan et al. (2011) found that the total VFA concentration (mmol/L) decreased by 17-23% with increasing levels of CT (20-60 g/kg DM), and Kondo et al. (2004) also indicated that the addition of green tea grounds (CT 23 g/kg DM of the diet) reduced total VFA production. The proportion of propionate increased by 4.3%; the acetate-to-propionate ratio decreased by 4.3%; and butyrate declined by 15.6 % when Sunphenon 30S-O was included at a dose of 50 g/kg DM. In support of our findings, Bhatta et al. (2009) reported that *in vitro* propionate production increased when the CT extract from either quebracho (*Schinopsis loentzii*) or mimosa (*Acacia mearnsii*) was added, and a decrease in the acetate-to-propionate ratio was observed when *Acacia mearnsii* extract was supplemented as a source of CT (Khiaosa-Ard et al., 2009). Conversely, the findings of Oskoueian et al. (2013) indicated that the inclusion of catechins did not have a significant effect on total VFA production.

4.4. Effects on in vitro nutrient degradability

Feeding forages containing CT have been reported to decrease ruminal protein degradation (Min et al., 2003) and to depress the feeding value of the diet (Hess et al. 2006), despite being effective in limiting methanogenesis. In our study, nutrient ruminal degradability was affected by the addition of Sunphenon 30S-O. There was a 1.6-7.3% reduction in IVDMD due to the inclusion of Sunphenon 30S-O in a dose-dependent manner. Similarly, the addition of Sunphenon 30S-O decreased IVOMD and IVRCPD by 3-9 % and 3-5 % respectively. Our finding was in agreement with the previous work by Oskoueian, et al., (2013), who indicated that addition of catechins decreased DM degradability significantly ($p<0.05$) and it is also consistent with Tan et al., (2011), who stated that in *vitro* DM degradability and nitrogen disappearance declined with increasing levels of CT. Previous work by Min et al., (2000, 2002), confirmed that CT in the diet reduced protein degradation and rumen NH_3-N concentrations. Study by Barman and Rai (2008), also confirmed that DM, OM and CP digestibility decreased with increasing levels of tannin 4 to

12% of the substrate. The in *vitro* data in our study suggests that optimum reductions of CH_4 (9.5%) without significant effect on nutrient degradability was obtained at lower and medium inclusion of Sunphenon 30S-O, 20-40g/kg DM of the substrate.

5. Conclusion

Sunphenon 30S-O contains standardized concentration of total catechin (20.5g/100g DM). EGCG and EGC are the major constituents of catachin contained in Sunphenon 30S-O, which could be responsible for influencing CH_4 emission and other rumen fermentation parameters. Addition Sunphenon 30S-O at different concentrations reduces CH_4 production, and this reduction was more pronounced in a dose dependent manner. Higher concentration of Sunphenon 30S-O (50 g/kg of the substrate), has shown to negatively affect *in vitro* nutrient digestibility, protozoa population and VFA concentration. Thus the findings of this study suggest that for optimum reduction of CH_4 without any negative effect on nutrient digestibility and other rumen fermentation parameters, relatively lower to medium concentration of Sunphenon 30S-O (20-40 g/kg DM) could be a possibility.

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Chapter Two

The effect of Sunphenon 30S-O on *in vivo* methane emission, nutrient intake and digestibility in sheep

Abstract

Sunphenon 30S-O is obtained from the leaves of traceable green tea (*Camellia sinensis*) and standardized for its catechin content (205 g/kg DM). This experiment was conducted to evaluate the effect of supplementing different concentrations of Sunphenon 30S-O on *in vivo* methane (CH_4) emission, nutrient intake and digestibility in sheep. Four Corriedale wethers with average body weight of 64.3±3.9 kg were arranged in 4x4 latin square design and fed a basal diet of Guinea grass (*Panicum maximum*) hay at maintenance level with four varying concentrations of Sunphenon 30S-O (0, 10, 25 and 40 g/Kg DM intake). The experiment was conducted over 84 days in four 21-day periods that consisted of 14 days of adaptation, five days of measurement and two 24-h runs in open circuit respiration chambers to measure gas exchange. The data were subjected to polynomial regression analysis. Dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) and Gross energy (GE) intake all declined linearly ($P<0.01$) and quadratically ($P<0.05$) with increasing concentration of Sunphenon 30S-O. Conversely, the apparent nutrient digestibility remained similar among treatments regardless of the concentration of Sunphenon 30S-O in the ration. *In vivo* CH_4 emission (l/kg digestible OM intake) declined linearly ($P<0.05$) by 7.4-13.5% with increasing concentrations of Sunphenon 30S-O. Urinary and CH_4 energy decreased linearly ($P<0.01$) from 17.4% to 11.2% and from 7.3% to 6.2% of the GE intake, respectively, with increasing supplement concentrations. The findings of this study indicated that the addition of Sunphenon 30S-O reduced *in vivo* CH_4 emissions without affecting total tract nutrient

digestibility, and energy and protein retention were not affected despite the reduction in total nutrient intake. Thus, to achieve optimum reduction of CH_4 emissions and the concomitant saving of dietary energy without any negative impacts on total-tract digestibility and nutrient balance, Sunphenon 30S-O supplementation up to 40 g/kg DM could be an option.

1. Introduction

Livestock is assumed to be responsible for 80% of the total agricultural greenhouse gas emission due to CH_4 release from enteric fermentation and manure handling (Olesen et al., 2006, Kristensen et al., 2011, Mihina et al., 2012). Methane emissions by ruminants is a loss of feed energy from the diet and represents inefficient utilization of the feed (Chagunda, et al., 2009) which is mainly related to the type and amount of feed consumed (Broucek, 2014; Shibata and Terada, 2010; Mukhtar and Ahmad 2000; Mitsumoto et al., 2005). Methane emission from ruminant livestock is currently estimated to be around 100 million tonnes each year. In future, this effect will become even more pronounced because ruminant production is increasing worldwide to meet ever increasing demand for animal product.

Thus modifying the diet composition is often regarded as one way to minimize ruminal CH_4 emission. Different plants and their parts have been identified as potential feed additives to lower CH_4 emission from cattle, sheep, and goats (Patra, 2010). Recently, natural plant products which are often inexpensive and environmentally safe have been introduced in CH_4 mitigation strategies. They could be superior feed additives to replace the ionophores and probiotics for controlling methanogenesis (Kamra et al., 2012). These compounds are not only able to suppress the CH_4 emission but also possess broad range of favorable effects on animal health. For instance, their major effects on gastrointestinal tract include improvement in digestibility, feed efficiency, and protection of dietary proteins from rumen microbial degradation, maintaining the gut microflora balance, gastric or liver damage prevention, reduction in gastrointestinal spasms, diarrhea, constipation, bloat, acidosis, and controlling gut pathogens (Durmic and Blache 2012).

Tannin reduces CH_4 due to their inhibitory effect upon methanogenesis, protozoa and other hydrogen-producing microbes (Patra and Saxena, 2010; Tavendale et al., 2005). Tea catechins (precurcer of condensed tannin) are a major group of polyphenolic flavonoids found in green tea. Green tea contains polyphenols consisting mainly of flavanol

(flavan-3-ol) monomers, which are referred to as catechins, the major component of green tea extract, have various physiological effects.

In vivo and *in vitro* study by Mitsumoto et al., 2005, Wang and Xu, 2013 and Zhong et al., 2009 indicated that green tea polyphenols improve growth performance, meat quality and shelf life due to their antioxidant properties in cattle, sheep and goats. Feeding diets containing 20% of the dietary DM as green tea waste silage to Holstein steers had no negative impact on ruminal fermentation, and increased plasma antioxidative activity and the concentration of vitamin E (Nishida et.al., 2006). However, their effectiveness in ruminant production has not been proved to be consistent and conclusive. There are contrasting reports of the effects of these phytoadditives on the rumen fermentation and rumen microbes probably depending upon the interactions among the chemical structures and levels of phytochemicals used. The results of the study reported by Oskoueian et al., 2013 indicated that catechin ($P < 0.05$) decreased DM degradability but no effect on gas production when it was included at the rate of 4.5% of the substrate on DM basis. Methane and VFA production was also not affected when catechin (4.5% w/w of the substrate) was incubated under *in vitro* condition (Oskoueian et.al., 2013).

Based on the recommendation of the previous studies there is a need to conduct more research to reach conclusive results. Therefore the present study was conducted to evaluate the effect of Sunphenon 30S-O supplementation on *in vivo* CH_4 emission, nutrient intake, digestibility and nutrient balance in sheep.

2. Materials and Methods

This experiment was conducted at Obihiro University of Agriculture and Veterinary Medicine, in accordance with the guidelines approval by the university animal use and care committee.

2.1. Sunphenon 30S-O.

Sunphenon 30S-O, which is standardized for catechin content (205 g/kg DM), was obtained from the leaves of traceable green tea (*Camellia sinensis*) via extraction by water infusion and decaffeination using approved food-grade solvents. Catechin is the only polyphenol present in Sunphenon 30S-O, which contains water soluble fibers as filler and whose chemical composition and major catechin components are presented in Table 1. Samples of Sunphenon 30S-O were purchased from Taiyo Kagaku Co., Ltd., Japan; Sunphenon® extracts are food grade and approved by the Japanese Foundation for Health and Nutrition for specific medical uses. They are certified organic and possess an excellent tea taste and maintain good stability in beverages.

Table 1. Chemical composition of experimental feeds

Item [a]	Guinea grass hay	Concentrate mixture	Sunphenon 30S-O
DM (g/kg)	956	958	944
OM (g/kg DM)	909	918	913
Ash(g/kg DM)	84.4	76.5	85.6
CP (g/kg DM)	147	165	137
EE (g/kg DM)	16.0	33.6	6.20
GE (MJ/kg DM)	17.1	16.7	15.9
NDF (g/kg DM)	609	232	20.0
ADF (g/kg DM)	303	78.3	9.00
ADL(g/kg DM)	36.2	35.6	2.00
(+)-catechin (g/kg DM)	-	-	3.00
EC (g/kg DM)	-	-	21.0
EGC (g/kg DM)	-	-	79.0
EGCG (g/kg DM)	-	-	84.0
ECG (g/kg DM)	-	-	18.0

[a]DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; GE: gross energy; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL acid detergent lignin; EC epi catechin; EGC epi galo catechin; EGCG epi galo catechin galate; ECG epi catechin galate

2.2. Animals, diets and supplements

Four Corriedale wether sheep with body weights of 64.25±3.86 kg were used in a 4×4 Latin square design. The wethers were kept in an individual metabolic cages equipped with a ventilated respiratory collection hood and fed a maintenance-level (55 g DM/kg $BW^{0.75}$/ day) basal diet of Guinea grass (*Panicum maximum*) hay twice daily (08:30 and 16:30), and all had free access to clean drinking water and a mineral block. The mineral block contains Fe, 1836 mg; Cu, 377 mg; Co, 66 mg; Mg, 1046 mg; Zn, 1235 mg; I, 77 mg; Se, 33 mg; Vit E, 5000 mg; Na, 962 g/1 kg DM basis). The treatments were as follows: 1. control (100% Guinea grass hay); treatments 2, 3 and 4 contained 10, 25 and 40 g Sunphenon 30S-O per kg DM, respectively, in addition to the amount contained in the control diet. Sunphenon 30S-O was thoroughly mixed with 50 g of concentrate mixture in each treatment to facilitate intake and to avoid loss; the control group was also supplemented with 50 g of the concentrate mixture.

2.3 Experimental procedure

The experiment lasted for 84 days, with each period consisting of 14 days of adaptation, 5 days of data collection and two 24 h runs for measurement of gas exchange in open respiration chambers. Samples of feed ingredients, refusal, feces and urine were analyzed for nutrient content following the standard procedures. The body weight was measured at the beginning and end of each period. Oxygen consumption, carbon dioxide and methane emission were quantitatively measured by an open circuit respiratory system using a hood over the wether's head as described by Takahashi et al. (1999). Data were collected and entered into a computer through an interface with the analysers at 1-min intervals and then automatically standardized at 0°C, 1013 hpa and zero water vapour pressure.

2.4. Calculation of energy balance

Total methane gas volume obtained from the open circuit respiratory system was converted to its gross energy (GE) value using the conversion factor 39.54 kJ/l (Brouwer, 1965). Digestible energy (DE) was calculated as the difference between energy intake and fecal energy, energy lost as methane was methane emitted in l/day * 39.54kJ/l (Brouwer,1965), Metabolizable energy (ME) was the difference between DE and the sum of energy in urine and CH_4, and energy retention (ER) was the difference between ME and Heat Production(HP). Heat production (kJ/day) was calculated using the equation; 16.18 O_2 (l/day) + 5.02 CO_2 (l/day) – 2.17 CH_4 – 5.99 N (g/day) (Brouwer, 1965). Energy retention was calculated as the difference between ME and HP.

2.5. Faeces *and urine collection and preparation*

Faeces and urine were collected for 5 days during each period, and the faecal samples from each treatment were thawed, bulked, mixed and sub-sampled. Sub-samples were dried at 60°C for 48 h in a forced-air oven and ground to pass through a 1-mm sieve for subsequent laboratory analysis. Urine was collected into buckets containing 100 ml of 100 ml/l (v/v) sulphuric acid to reduce the pH below 3.0 and to prevent bacterial degradation of N compounds. Approximately 50 ml/l of the urine sample was sub-sampled and stored at -20 °C until the nitrogen analysis.

2.6. *Laboratory analysis*

Samples of Guinea grass hay, concentrate mixture and Sunphenon 30S-O were analysed for DM by drying at 135^{o}C for 2 h (930.15), OM and total ash (942.05), and ether extract (EE) (920.39) following the procedures of AOAC, 1995. Nitrogen was determined by the Kjeldahl method (984.13) (AOAC, 1995) using an electrical heating digester (FOSS tecatorTM Digester, Tokyo, Japan) and an automatic distillation apparatus (FOSS

kjeltecTM 2100, Tokyo, Japan), and then, crude protein (CP) was determined as N × 6.25. Neutral detergent fibre (NDF) was determined according to the method described by Van Soest, *et al.,* (1991), and it was estimated without amylase and expressed inclusive of residual ash. Acid detergent fibre (ADF) and lignin were also determined following the procedure of Van Soest, *et al.,* (1991). ADF was expressed as inclusive of ash. Lignin was determined by the solubilization of cellulose with sulphuric acid. The Gross Energy (GE) content of the samples was analysed in a Shimadzu auto-calculating bomb calorimeter (CA-4AJ, Shimadzu Corporation, Japan).

The components of total catechins in Sunphenon 30S-O were analysed by Japan Food Research Laboratories using high-performance liquid chromatography (HPLC). EC, EGC, EGCG, ECG and (+)-catechin were separated by a reverse phase mechanism on a C18 column with water, methanol and 0.02 mol/l phosphate buffer (pH=3.0) mobile phase gradient. EC was detected and quantified by fluorescence with excitation at 280 nm and measured at 315 nm with a flow rate of 1.0 ml/min. EGC, EGCG and ECG were detected by ultra-violet light at 270 nm with 1 ml/min flow rate. (+)-Catechin was separated by Atlantis T3 2.1 mm*150 mm column with acetonitrile, acetic acid and water linear gradient ionization as a mobile phase.

2.7. Statistical analysis

Data obtained from the *in vivo* study were subjected to ANOVA in a 4 x 4 Latin square design using a polynomial regression analysis (REG procedure) available in SAS (2010) with the model: $Yij = \mu + Ti + eij$, where *Yij* is the dependent variable; μ is the overall mean; *Ti* is the fixed treatment effect; and *eij* is the residual. The experimental unit was the individual animal. Differences among the means were identified using Tukey's multiple comparisons, and effects were considered significant when $P<0.05$ while trends were discussed at $0.05<P<0.10$. The standard error of the means was determined using the least squares means procedure (lsmeans option) in SAS (2010). The relationship was

analyzed using linear quadratic and cubic regression (PROC REG).

3. Result

3.1. Chemical composition

Sunphenon 30S-O contains standardized concentration of catechin (20.5 g/100 g). EGCG and EGC are the major constituents (80%) of the total catechin in Sunphenon 30S-O. The CP and GE content of Sunphenon 30S-O are comparable to that of Guinea grass hay and concentrate mixture where as the fiber content (NDF and ADF) is negligible (Table 1).

3.2. Nutrient intake, digestibility and loss

Increasing the concentration of Sunphenon 30S-O to 40 g/kg DM resulted in a linear ($P<0.01$) and quadratic ($P<0.05$) decrease in DM, OM, CP, NDF and ADF intake. The effect of Sunphenon 30S-O on DM and OM intake are indicated in Fig 1. Nutrient digestibility (DM, OM, CP, NDF and ADF) was not influenced by supplementation of green tea extract (Table 2). Gross energy intake (MJ/d) and DE intake (MJ/d) were reduced linearly ($P<0.01$) with increasing concentrations of Sunphenon 30S-O, but ME intake was not affected ($P>0.05$) (Table 3). Energy losses through urine and CH_4 were reduced linearly ($P<0.01$) with increasing concentrations of Sunphenon 30S-O, but energy loss through the faces was not affected ($P>0.05$) Fig 3. Heat production and ER did not differ among treatments ($P>0.05$). Crude protein loss through urine was reduced linearly ($P<0.01$), but there was no influence on CP loss through faces ($P>0.05$) Fig 4. Crude protein retention was not affected ($P>0.05$) by the addition of Sunphenon 30S-O (Table 4).

3.3. *Effect on methane emission, energy intake and loss*

In vivo CH_4 emissions (L/d) decreased linearly ($P<0.01$) in a dose-dependent manner when Sunphenon 30S-O was added in the diet of sheep (Table 3). Methane

emissions (l/kg digestible OM intake) also decreased linearly ($P<0.05$) as the level of Sunphenon 30S-O increased. Carbon dioxide production (L/d) decreased linearly ($P<0.05$) and quadratically ($P<0.05$) as the level of supplementation increased. Comparison of CH_4 emissions in terms of digestible OM and digestible DM are indicated in Fig 2.

Table2. Intake and digestibility of nutrients by sheep supplemented with different concentration of Sunphenon 30S-O

Item[1]	Sunphenon 30S-O concentrations (g/kg DM)				SEM	Contrasts[2]		
	0	10	25	40		L	Q	C
DM								
intake (g/d)	1016[a]	1014[a]	971[a]	857[b]	12.24	<.001	0.041	0.812
digested (g/d)	637[a]	636[a]	591[ab]	532[b]	8.728	0.001	0.135	0.741
digestibility	0.63	0.63	0.63	0.61	0.007	0.688	0.554	0.394
OM								
intake (g/d)	923[a]	922[a]	882[a]	779[b]	11.13	<.001	0.041	0.812
digested (g/d)	589[a]	589[a]	544[ab]	493[b]	7.948	<.001	0.150	0.618
digestibility	0.64	0.64	0.62	0.64	0.006	0.718	0.466	0.264
CP								
intake (g/d)	150[a]	149[a]	143[a]	126[b]	1.803	<.001	0.041	0.812
digested (g/d)	120[a]	120[a]	113[a]	99.4[b]	1.414	<.001	0.039	0.981
digestibility	0.80	0.80	0.79	0.79	0.004	0.169	0.85	0.49
NDF								
intake (g/d)	619[a]	618[a]	591[a]	522[b]	7.46	<.001	0.041	0.812
digested (g/d)	405[a]	407[a]	373[ab]	337[b]	5.350	<.001	0.103	0.515
digestibility	0.66	0.66	0.63	0.65	0.006	0.486	0.605	0.170
ADF								
intake (g/d)	308[a]	307[a]	294[a]	260[b]	3.710	<.001	0.041	0.812
digested (g/d)	178[a]	177[a]	165[ab]	148[b]	2.655	<.001	0.186	0.847
digestibility	0.58	0.58	0.58	0.56	0.007	0.716	0.570	0.557

[1]DM: dry matter; OM: organic matter; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre;

[a-c]Means within a raw with different superscripts differ($P<0.05$)

[2] L = linear, Q = quadratic, C = cubic.

Table3. Methane emission and energy balance by sheep supplemented with different concentrations of Sunphenon 30S-O

Item[1]	Sunphenon 30S-O concentrations (g/kg DM)				SEM	Contrasts[2]		
	0	10	25	40		L	Q	C
Methane emission								
CH_4 (l/d)[1]	34.7[a]	32.3[ab]	30.2[b]	24.4[c]	0.484	<.001	0.120	0.379
CH_4 (g/d)	24.9[a]	23.1[ab]	21.7[b]	17.5[c]	0.347	<.001	0.120	0.379
CH_4 E (MJ/d)	1.27[a]	1.18[ab]	1.12[b]	0.91[c]	0.031	<.001	0.372	0.494
CH_4 (l/kg DMI)	34.2[a]	31.8[a]	31.2[ab]	28.5[b]	0.492	0.001	0.879	0.448
CH_4 (g/kg DMI)	24.5[a]	22.8[a]	22.3[ab]	20.4[b]	0.352	0.001	0.879	0.448
CH_4 (g/kg DDMI)	39.3[a]	36.5[ab]	38.1[ab]	34.2[b]	0.601	0.025	0.667	0.099
CH_4 (l/kg DDMI)	54.9[a]	51.0[ab]	53.3[ab]	47.7[b]	0.84	0.025	0.667	0.099
CH_4 (g/kg DOMI)	42.5[a]	39.4[ab]	41.4[ab]	36.8[b]	0.647	0.022	0.622	0.074
CH_4 (l/kg DOMI)	59.4[a]	55.0[ab]	57.8[ab]	51.4[b]	0.903	0.022	0.622	0.074
Energy balance (MJ/d)								
GE Intake	17.3[a]	17.3[a]	16.6[a]	14.6[b]	2.090	<.001	0.041	0.812
Fecal	6.27	6.45	6.36	5.53	0.142	0.105	0.109	0.733
DE	11.1[a]	10.9[a]	10.2[ab]	9.10[b]	1.481	<.001	0.181	0.995
Methane	1.27[a]	1.18[ab]	1.11[b]	0.91[c]	0.291	<.001	0.422	0.592
Urinary	3.01[a]	2.28[ab]	2.04[b]	1.63[b]	0.102	<.001	0.496	0.533
ME	7.92	8.44	8.04	7.37	1.841	0.275	0.150	0.722
HP	7.75	8.24	7.90	7.26	0.118	0.414	0.755	0.681
ER	0.17	0.20	0.14	0.12	0.097	0.491	0.860	0.995

[1]CH_4: methane; DMI: dry matter intake; DDMI: digestible dry matter intake; DOMI: digestible organic matter intake; GE: gross energy; DE: digestible energy; ME: metabolizable energy; HP: heat production; ER: energy retained

[a-c]Means within a raw with different superscripts differ($P<0.05$)

[2] L = linear, Q = quadratic, C = cubic

Table4. The effect of Sunphenon 30S-O on urinary and fecal crude protein losses

Item[1]	Sunphenon 30S-O concentrations (g/kg DM)				SEM	Contrasts[2]		
	0	10	25	40		L	Q	C
Intake (g/d)	150[a]	149[a]	143[a]	126[b]	1.803	<0.001	0.041	0.812
Feces (g/d)	29.7	29.8	30.2	26.9	0.671	0.233	0.252	0.562
Urine (g/d)	53.5[a]	44.5[ab]	41.6[ab]	31.4[b]	1.880	0.004	0.895	0.482
CP retained (g/d)	66.4	75.0	71.2	67.9	2.234	0.976	0.237	0.561

[1] CP： crude protein

[a-b]Means within a raw with different superscripts differ(P<0.05)

[2] L = linear, Q = quadratic, C = cubic

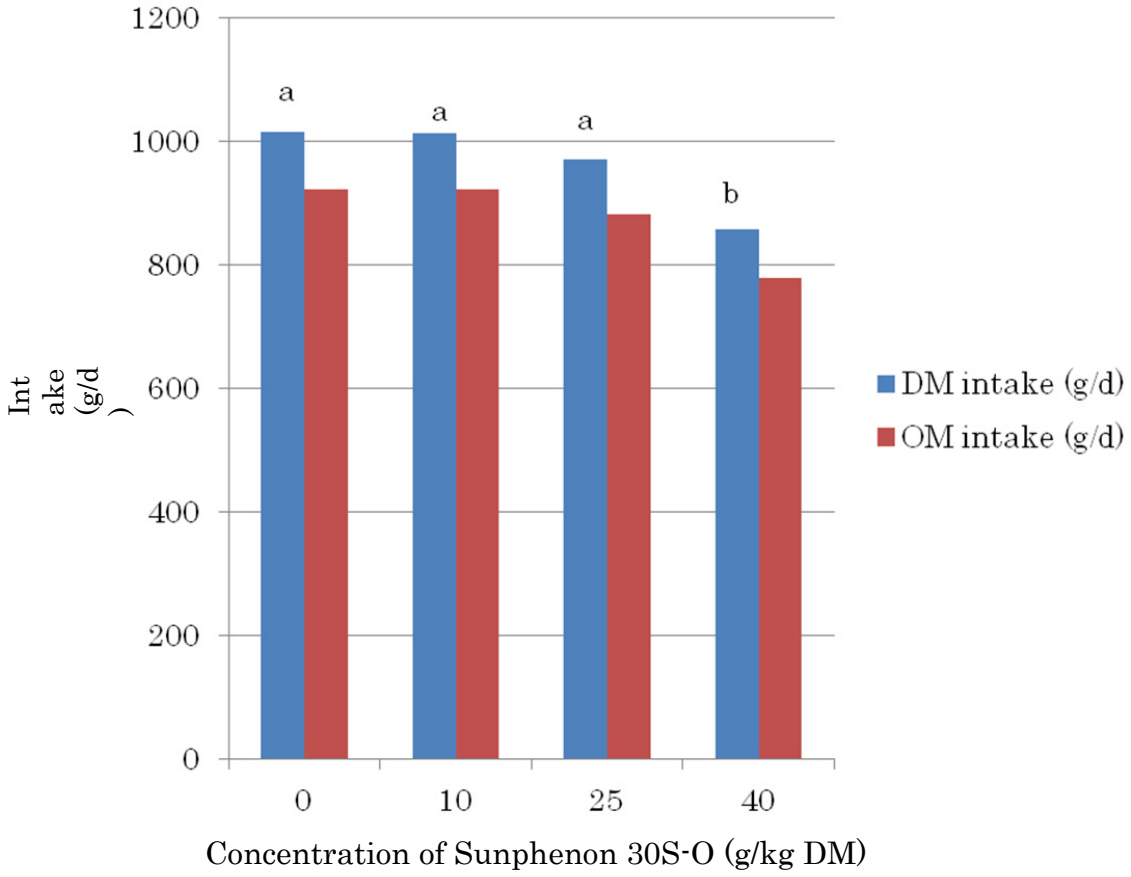

Fig1. Effect on Sunphenon 30S-O on dry matter and organic matter intake
[a-b]Means with different superscripts differ($P<0.05$)

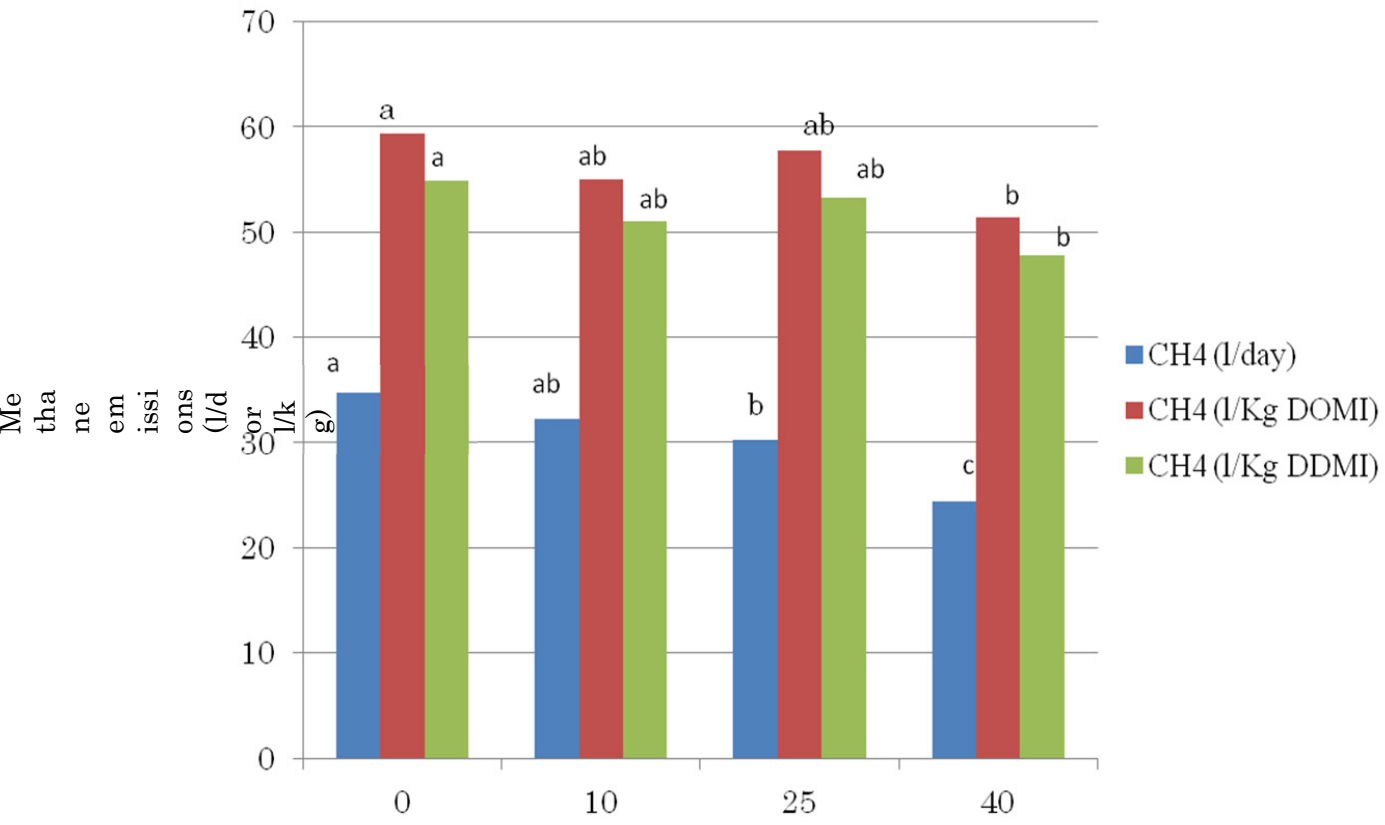

Fig2. Effect of Sunphenon 30S-O on *in vivo* methane emission
[a-c]Means within different superscripts differ($P<0.05$)

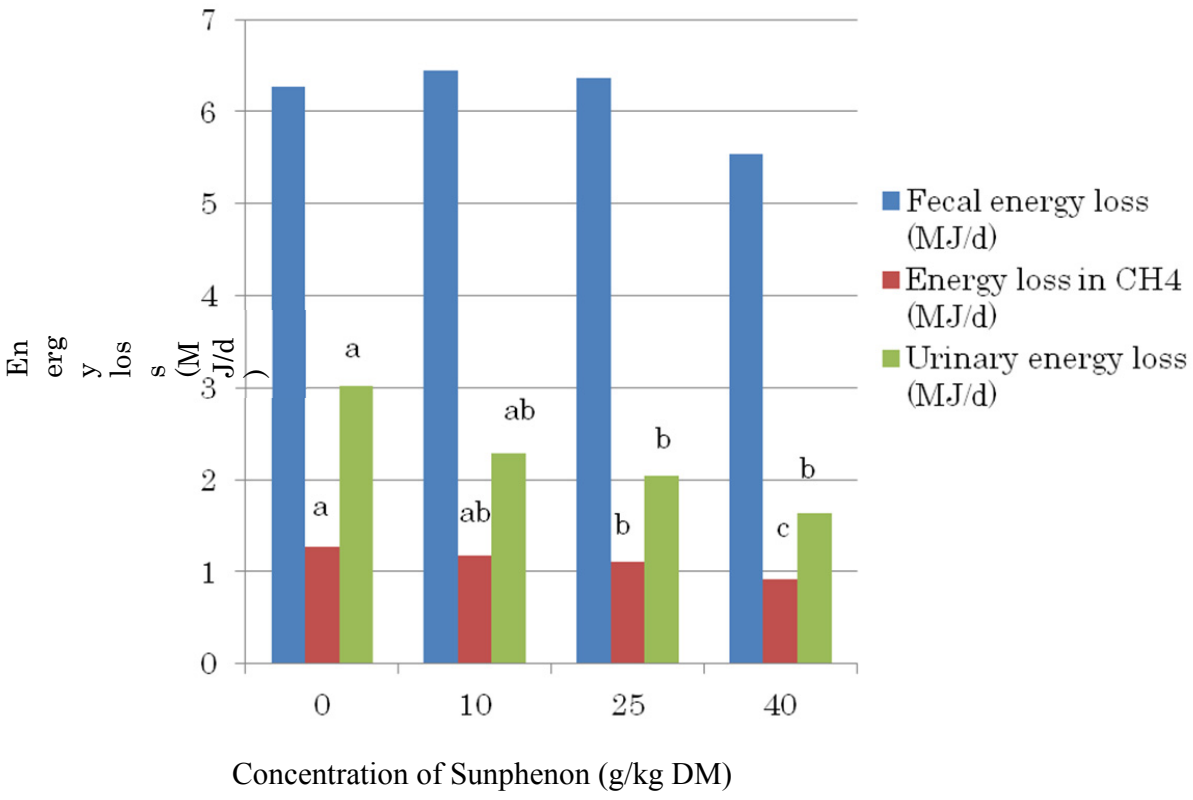

Fig3. Effect of Sunphenon 30S-O on energy loss through feces, urine and methane
[a-c]Means with different superscripts differ($P<0.05$)

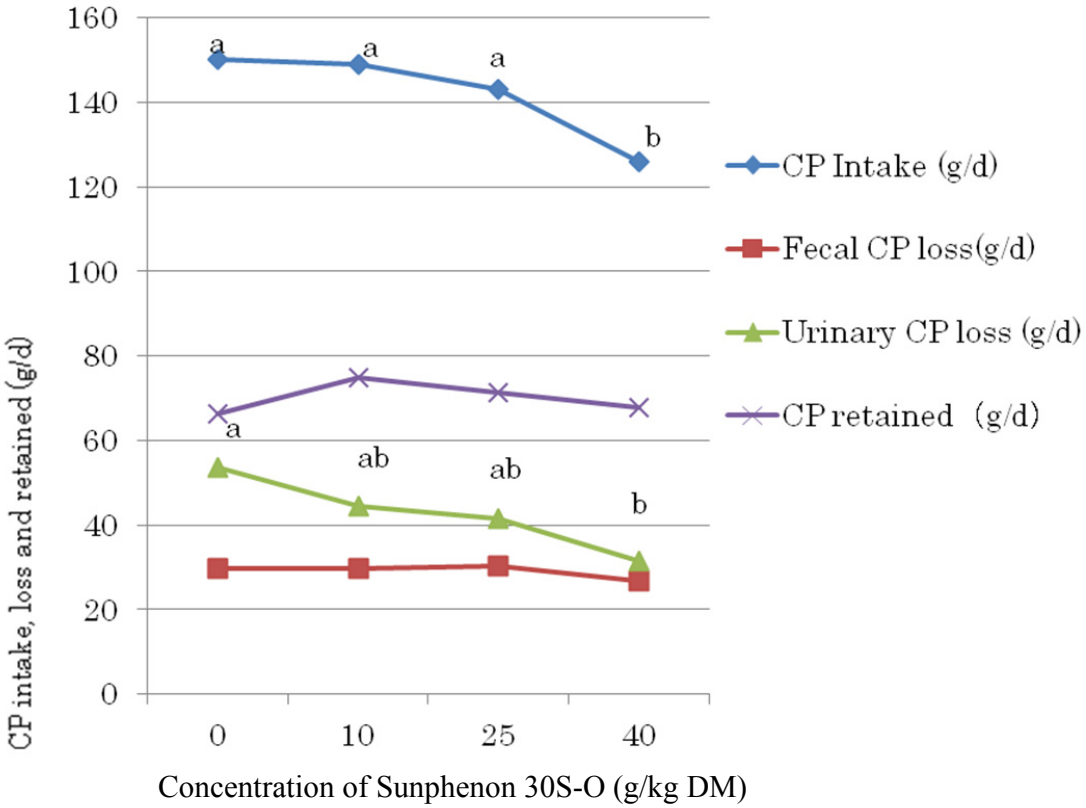

Fig4. Effect of Sunphenon 30S-O on *in vivo* crude protein intake, loss and retained (g/d)
[a-b]Means with different superscripts differ($P<0.05$)

4. Discussion

4.1. *Nutrient intake and digestibility*

In small ruminants, the level of food intake is inversely related to the concentration of CT in the food (Landau et al., 2002; Silanikove et al., 1994; Silanikove et al., 1997a). In the present experiment, daily nutrient intake (DM, OM, CP, NDF or ADF) was not affected by supplementation with 10-25 g of Sunphenon 30S-O per kg of DM, but when the concentration was increased to 40 g/kg DM, the intake of DM, OM, NDF and ADF was reduced by 15.6% and that of CP by 13.9%. Dschaak et al. (2011) reported that supplementation with CT extract (30 g/kg DM) decreased DM, OM, CP, NDF, and ADF intake. The presence of catechin (a precursor of CT) in Sunphenon 30S-O could have affected nutrient intake due to its astringency as in previous studies in which CT from quebracho (*Aspidosperma quebracho*) was shown to negatively affect the intake of Holstein heifers (Landau et al., 2000). Additionally, the relative amounts of consumed plant secondary compounds affect intake and forage preference of herbivores (Mote et al., 2007). Conversely, the inclusion of Sunphenon 30S-O at different concentrations did not affect the total-tract digestibility of DM, OM, CP, NDF and ADF, and the overall CP digestibility coefficient was 0.80, 0.80, 0.79 and 0.79 for concentrations of 0, 10, 25 and 40 g/kg DM of Sunphenon 30S-O, respectively. This is consistent with the findings by Dschaak et al. (2011), who found that although supplementation with CT extract in the diet (30 g/kg of DM) decreased feed intake, total–tract digestibility of DM, OM, CP and ADF was not affected. Metabolizable energy intake, HP and ER were also not affected ($P>0.05$), which could be due to the increased nutrient utilization efficiency resulting from the addition of Sunphenon 30S-O. A previous study by Hess et al. (2006) indicated that, despite increased ($p<0.01$) total energy losses, tannins affected neither ($p>0.05$) energy expenditure nor body energy retention.

Feeding forages containing condensed tannin (CT) have been reported to decrease ruminal protein degradation and depress the feeding value of the diet. Inclusion of CT (32 g/kg DM) from *Lotus corniculatus* reduced nitrogen degradability by 10% (Min et al., 2002a), and the addition of CT (25 g/kg DM) from the bark of *Acacia mearnsii* reduced ($P<0.05$) apparent digestibility of all nutrients except hemicelluloses (Hess et al., 2006). In our *in vitro* study, ruminal DM and OM degradability declined by 2-7% and 3-9%, respectively, due to the inclusion of Sunphenon 30S-O (20-50 g/kg DM). This finding agrees with previous work by Oskoueian et al. (2013), who indicated that a 4.5% (w/w) dose of catechins decreased DM degradability ($p<0.05$) by 6.7% compared to the control. Our findings are also consistent with those of Tan et al. (2011), who stated that *in vitro* DM degradability and N disappearance declined by 7% and 15%, respectively, with the addition of CT (30 g/kg DM).

4.2. The effect of Sunphenon 30S-O on methane emissions and energy balance

The inclusion of natural feed additives should be considered from the perspective of their effect on environmental safety and nutrient utilization efficiency. The findings of our *in vivo* study indicated that Sunphenon 30S-O (20-40 g/kg DM) supplementation decreased CH_4 emissions (l/kg digestible OM intake) by 7.4-13.5% compared to the control. It has been indicated that supplementation with *Acacia mearnsii* tannin (25 g/kg dietary DM) decreased CH_4 emissions by 0.13 of GE intake (Hess et al., 2006). Similarly, a study by Tan et al. (2011) indicated that CT extracts from *Leucaena leucocephala* hybrid-Rendang (20-60 g/kg DM of the diet) reduced CH_4 emissions by 0.33-0.63 of the DM. Methane emissions from dairy cows were reduced by 0.23 of digestible DM when fed silage made from *Lotus corniculatus* (CT 26 g/kg DM) compared to silage from pasture (Woodward et al., 2001). In the present study, 1 mol of catechin in Sunphenon 30S-O (1.0% of the substrate on a DM basis) reduced the emission of CH_4 by 1.8 mol while the findings of Becker et al. (2013) suggested that catechins decreased CH_4 production in a

dose-dependent manner, where 1.0 mol of catechin prevented the emission of 1.2 mol of CH_4. In the control animals, energy wasted as urine and CH_4 represented 17.4 and 7.3% of the GE intake, respectively, where it only represented 11.2 and 6.2% of the GE intake in the treated animals (supplemented with Sunphenon 30S-O at the dose of 40 g/kg DM). This indicates that, compared to the control, the urinary and CH_4 energy emissions decreased significantly, but the basal metabolism remained unchanged because GE intake was reduced by 16%.

Energy retention was not affected by the addition of different concentrations of Sunphenon 30S-O. Dietary energy loss through CH_4 emissions by sheep was 0.06 – 0.07 of the total GE intake, which agrees with the findings by Sauvant and Giger-Reverdin (2007), who reported CH_4 losses of 0.06 – 0.07 of GE with a lower proportion of concentrate in the diet. Animut et al. (2008) also reported CH_4 emissions by meat goats of 0.09 of GE intake with ad libitum consumption of sorghum-Sudan grass.

4.3. The effect of Sunphenon 30S-O on protein utilization efficiency

In this study, protein intake was reduced by up to 0.16 of the total CP intake at the highest level of Sunphenon 30S-O supplementation (40 g/kg DM). Crude protein loss accounted for 0.46–0.57 of the total CP intake, and most of the loss (0.54–0.64 of the total CP loss) was through urine, which was reduced by 17-41% under supplementation. Despite the reduction in total CP intake, retained CP was not affected by Sunphenon 30S-O supplementation, and this could be attributed to the effect of supplementation, which reduced CP loss through urine and increased CP absorption efficiency in the lower tract. This is consistent with Priolo and Ben Salem (2004), who stated that low concentrations of CT appear to reduce protein degradation in the rumen and enhance the availability and absorption of amino acids from the small intestine. Moderate levels of CT (20-40 g/kg DM) bind to protein in the rumen to form a CT-protein complex but then dissociate, and the protein becomes available in the abomasums (Barry et al., 2001). Makkar (2003) also

indicated that CT protects dietary protein from rumen microbial degradation and increases the supply of amino acids to the intestine for greater absorption.

5. Conclusion

EGCG and EGC are the major constituents of catechin present in Sunphenon 30S-O, which could be responsible for influencing CH_4 emission and other rumen fermentation parameters. Our *in vivo* study suggests that the addition of Sunphenon 30S-O reduced CH_4 emissions in a dose-dependent manner. Although supplementation reduced feed intake in sheep, the total-tract digestibility of nutrients was not affected regardless of the Sunphenon 30S-O concentrations in the diet. Thus, to achieve optimum CH_4 reduction and save dietary energy without any negative effect on whole-tract nutrient digestibility, Sunphenon 30S-O supplementation up to 40 g/kg DM of the diet could be a possible option.

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Chapter Three

Effects of Euglena *(Euglena gracilis)* supplemented to diet (forage: concentrate ratios of 60:40) on the basic ruminal fermentation and methane emissions in *in vitro* condition

Abstract

An *in vitro* study was conducted to investigate the effect of different concentrations of Euglena (*Euglena gracilis*) on methane (CH_4) production, dry matter (DM) digestibility, volatile fatty acid (VFA） and ammonia-N (NH_3-N) concentrations as well as on the protozoa population. The treatments considered were Euglena at concentrations of 0.0, 50, 100, 200, 400 and 1000 g/kg dry matter (DM) of the substrate (60:40 forage: concentrate ratio) incubated for 24 and 96 h using an *in vitro* continuous gas production and *in vitro* two-stage digestion procedure, respectively. The data were subjected to polynomial regression analysis. Methane emissions (ml/g DM) decreased at an increasing rate, generally with increasing concentration of Euglena but also exhibited quadratic ($P<0.001$) and cubic ($P<0.001$) effects while NH_3-N (mg/ml) concentration increased at an increasing rate (linear $P<0.001$; quadratic $P=0.001$; cubic $P=0.024$). Total VFA concentration (mmol/l) decreased significantly ($P<0.001$), when the substrate was totally replaced by Euglena. There was a linear ($P<0.001$) and cubic ($P=0.047$) reduction in protozoa population as the concentration of euglena increased. *In vitro* DM digestibility was improved (linear $P=0.003$; quadratic $p=0.04$; cubic $P<0.001$). These findings suggest that Euglena at concentration of 100 g/kg DM reduce CH_4 emissions by 9.1% and improve DM digestibility by 15.26%. However, when the concentration of Euglena increases, while further reducing CH_4 emissions, have negative effect on NH_3-N concentration, protozoa population and VFA concentration.

1. Introduction

The contribution of livestock production towards environmental pollution is becoming of great concern because of the emissions of greenhouse gases, such as CO_2, CH_4 and ammonia. In addition, the production of CH_4 during the enteric fermentation of feeds in the rumen is correlated with the loss of gross energy (GE) from the consumed feed (Szumacher-Stabel and Cieslak, 2012). Thus, identifying alternative solutions to this major constraint is a concern of both environmental protection and nutrient utilization. The efficiency of ruminal fermentation can be facilitated by modifying the feeding system using natural feed additives, thereby reducing the emission of greenhouse gases and enhancing the efficiency of nutrient utilization.

Microalgae are one of the most promising biological resources, as these organisms are rich sources of vitamins, minerals, proteins, polyunsaturated fatty acids, antioxidants, etc. (Pulz and Gross, 2004) and can be used to enhance the nutritional value of animal feed, reflecting the well-balanced chemical composition of these microphytes. The inclusion of microalgal biomass in small quantity positively affects the physiology of animals, as antibacterial action, improve gut function, feed conversion and reproductive performance have been reported (Harel and Clayton, 2004). A number of nutritional studies have demonstrated the suitability of microalgae biomass as a potential substitute for conventional protein supplements, such as soybean and fish meal (Dajana et al., 2013).

Carbon dioxide fixation through *Euglena gracilis* is effective and economical (Chae et al., 2006), thereby lowering the greenhouse effect and climate changes through the absorption of increasing CO_2 emissions in the atmosphere. Microalgae can be cultivated in areas unsuitable for other plants with several fold higher production and can effectively utilize and remove pollutants (e.g., nitrogen and phosphorus) from water (Gouveia et al., 2008). Thus Euglena, due to its rich source of fatty acid, protein and other biologically active compounds, inclusion of these micro algae in the ration of ruminants may influence

the emissions of CH_4, rumen fermentation and efficiency of nutrient utilization. As far as our knowledge is concerned, there is no information available on the effect of Euglena on CH_4 emissions. Therefore, our objective was to investigate the effect of different concentrations of Euglena on *in vitro* CH_4 emissions DM digestibility, VFA concentration, protozoa population and NH_3-N concentration.

2. Materials and Methods

2.1. Euglena (Euglena gracilis).

Euglena *(Euglena gracilis), powder* form with 100% purity, was obtained from Euglena Co. Ltd., Japan. The chemical composition of Euglena and the substrate (grass hay and concentrate mixture) are indicated in Table 1.

2.2. Rumen fluid sampling

Two ruminally fistulated non-lactating Holstein cows (average of 600 kg BW) were used as rumen fluid donors. The cows were maintained on a daily diet of 10 kg Orchard grass hay (Organic matter (OM), 980 g/kg; Crude protein (CP), 132 g/kg; Neutral detergent fiber (NDF), 701 g/kg; Acid detergent fiber (ADF), 354 g/kg; Lignin, 40 g/kg and GE, 18.02 MJ/kg; DM basis), with free access to clean drinking water and mineral block (Fe, 1836 mg; Cu, 377 mg; Co, 66 mg; Mg, 1046 mg; Zn, 1235 mg; I, 77 mg; Se, 33 mg; Vit E, 5000 mg; and NaCl, 962 g/1 kg). The rumen fluid from the two cows was sampled prior to morning feeding using a vacuum line and strained through a woven nylon cloth into a thermos flask, pre-heated to 39˚C with hot water. In the laboratory, the samples were pooled in equal proportions and continuously flushed for one hour with CO_2. The inoculum was immediately dispensed after preparation. Animal management and sampling procedures were approved through the animal care and use committee of Obihiro University of Agriculture and Veterinary Medicine.

Table 1：Chemical composition (g/kg DM) of experimental feeds

	Euglena	Klein grass hay	Concentrate mixture	Euglena concentrations (g/kg DM)						SEM
				0	50	100	200	400	1000	
Dry matter (g/kg)	960	956	958	957	957	957	957	958	960	0.358
Organic matter	961	908	918	912^{b}	914^{b}	916^{b}	921^{b}	929^{ab}	961^{a}	0.803
Ash	34.5	84.4	76.5	81.2^{a}	79.1^{a}	77.0^{ab}	72.8^{b}	64.4^{c}	34.5^{d}	0.147
Crude protein	240	147	164	154^{d}	158^{d}	162^{cd}	169^{c}	185^{b}	240^{a}	0.301
GE (MJ/kg DM)[1]	12.8	17.1	16.7	16.9^{a}	16.7^{a}	16.5^{ab}	16.1^{ab}	15.4^{b}	12.8^{c}	0.029
Ether extract	138	15.9	33.6	23.0^{d}	28.2^{d}	33.4^{cd}	48.8^{c}	64.7^{b}	138^{a}	0.353
NDF[2]	0.0	609	232	458^{a}	447^{a}	435^{ab}	412^{b}	366^{c}	0.0^{d}	1.979
ADF[3]	0.0	303	78.3	213^{a}	209^{a}	205^{a}	198^{ab}	182^{b}	0.0^{c}	1.102

[1]GE = gross energy; [2]NDF = neutral detergent fiber; [3]ADF = acid detergent fiber;
[a-d]means within a raw with different superscripts differ ($P<0.05$)

2.3. Experimental treatments and in vitro fermentation

The experimental samples were oven-dried at 60°C for 48 h and stored under dry and cool conditions in sealed containers prior to use. Six treatments were prepared containing different concentrations of Euglena, Klein grass (*Panicum coloratum*) hay and concentrate mixture. The following treatments were evaluated: 6 g of Klein grass hay + 4 g concentrate (Control, T1); 6 g of Klein grass hay + 3.5 g of concentrate + 0.5 g Euglena (T2); 6 g of Klein grass hay + 3 g of concentrate + 1 g of Euglena (T3); 6 g of Klein grass hay + 2 g of concentrate + 2 g of Euglena (T4); 6 g of Klein grass hay + 4 g of Euglena (T5) and 10 g of Euglena (T6). The effects of each treatment (10 g of DM) on CH_4 production, VFA concentration, NH_3-N concentration, pH, oxidation reduction potential (ORP) and protozoan population were tested *in vitro* for 24 h at 39°C using a continuous gas quantification system as previously described (Sar et al., 2005). The buffer was prepared according to McDougall (1948), sterilized by autoclaving and flushed with CO_2 for 1 h prior to dispensing into fermentation vessels. Fermentation was continued for 24 h at 39˚C. Rumen fluid was added to buffer at a ratio of 1:4. The gas output from each fermentation vessel was measured for 10 minutes at 30-min intervals. Samples of the incubation medium were collected at the end of each incubation period (24 h) and stored at -20°C for NH_3-N and VFA analysis. Then, the contents were discharged, and the fermentation vessels were thoroughly washed and autoclaved. The experiment was repeated four times on separate days, with treatments randomly assigned to the four fermentation vessels for each incubation period.

2.4. Analysis of methane and volatile fatty acids

The CH_4 production from each fermentation vessel was continuously measured using auto infrared CH_4 (EXA IR, Yokogawa Electric Corporation, Tokyo, Japan) analyzers, installed in an *in vitro* continuous gas quantification system (Takasugi

Seisakusho Co. Ltd., Tokyo, Japan). The components and total VFA were determined through gas chromatography (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (ULBON HR-52, 0.53 mm ID × 30 m, 3.0 μm) using 2-ethyl-n-butyric acid as an internal standard. The samples were prepared for analysis according to Sar et al. (2005). The pH and ORP of the fermentation media were monitored in each vessel at 1-min intervals (TS mk-250, Takasugi-ss Co. Ltd., Japan). All data were pooled and stored on a computer through an interface using the analyzers.

2.5. In vitro dry matter and organic matter digestibility

In vitro nutrient digestibility was estimated using the two-stage digestion technique according to Tilley and Terry (1963). Duplicate samples of 0.3 g of Klein grass hay + 0.2 g of concentrate (control, T1), 0.3 g of Klein grass hay + 0.175 g of concentrate + 0.025 g Euglena (T2), 0.3 g of Klein grass hay + 0.15 g of concentrate + 0.05 g of Euglena (T3), 0.3 g of Klein grass hay + 0.1 g of concentrate + 0.1 g of Euglena (T4), 0.3 g of Klein grass hay + 0.2 g of Euglena (T5) and 0.5 g of Euglena (T6) were weighed and placed into a 100-ml plastic bottle, and 40 ml of McDougall's buffer (McDougall, 1948) was added to each bottle and pre-warmed to 39°C. Subsequently, 10 ml of strained rumen fluid was dispensed into each bottle and sealed under continuous supply of CO_2 gas. The mixture was incubated at 39˚C for 48 h, with occasional careful shaking. The acid-pepsin solution was subsequently added, and the contents were incubated for another 48 h at 39˚C. Then the contents were filtered through pre-weighed Gooch crucibles, and the residual DM was determined. The loss in weight was determined as *in vitro* dry matter digestibility (IVDMD), followed by ashing the residues to estimate *in vitro* organic matter digestibility (IVOMD).

2.6. Amino acid and fatty acid composition of Euglena

Amino acid and fatty acid composition of Euglena sample was analyzed by Japan Food Research Laboratories, Japan. The amino acid composition except for tryptophan was carried out by an automated amino acid analyzer (JLC-500/V, JEOL ltd. Japan; Column, LCR-6 with 4 mm x 120 mm ID, JEOL, Co. Ltd., Japan). Tryptophan was analyzed by high performance liquid chromatography (HPLC, LC-20AD, Shimadzu, Co. Ltd., Japan; Column, CAPCELL PAK C18 AQ, 4.6 mm ID x 250 mm, Shiseido Co. Ltd., Japan; detector, Flourospectro photometer (RF-20Axs, Shimadzu, Co. Ltd., Japan). Mobile phase consisted of perchloric acid and methanol (80:20). The flow rate was 0.7 ml/min and the fluorescence excitation was at 285 nm and 40 ^{o}C.

Fatty acid composition of Euglena was determined by Gas chromatography, GC-1700, Shimadzu Co. Ltd., Japan equipped with FID. The fatty acids were separated on 30 m x 0.25 mm ID, DB-23 capillary column. Helium was used as a carrier gas at a flow-rate of 1.5 ml/min with split less injection at 250^{o}C and the detector temperature was 250^{o}C.

2.7. Chemical analysis

Samples of Euglena, Klein grass hay and concentrate were analyzed for DM after drying at 135˚C for 2 h (930.15), OM and total ash (942.05), and ether extract (EE) (920.39) according to the procedures of the Association of Official Analytical Chemists (AOAC) (1995). Nitrogen was determined through the Kjeldahl method (984.13) (AOAC, 1995) using an electrical heating digester (FOSS Tecator^{TM} Digestor, Tokyo, Japan) and an automatic distillation apparatus (FOSS Kjeltec^{TM} 2100, Tokyo, Japan), and CP was determined as N × 6.25. The NDF and ADF content were determined according to the method of Van Soest, et al. (1991). Both NDF and ADF were estimated without amylase and expressed inclusive of residual ash. The GE content of the samples was analyzed using

a Shimadzu auto-calculating bomb calorimeter (CA-4AJ, Shimadzu Co. Ltd., Japan). The NH_3-N concentrations were analyzed according to Conway and O' Malley (1942).

2.8. Statistical analysis

The data were analyzed using REG procedure of SAS (2010). The treatments with different concentration of Euglena were included as a fixed effect and the fermentation vessels/bottles as random effects in the model. *In vitro* digestibility was completed in four runs, with each sample replicated four times in a single run. The replication average within a run was considered as a statistical unit. In cases of *in vitro* gas production, each treatment was incubated four times in different runs (statistical replicates). The total effects included in the model for each variable were four replications and six treatments. Linear, quadratic and cubic contrasts of the treatment means were assessed. Differences among the means were identified using Tukey's multiple comparisons. The effects were considered significant at $P<0.05$ and trends were discussed at $0.05<P<0.10$

3. Results

3.1. Chemical composition of Euglena

The chemical composition of the experimental feeds used in the present study indicated that Euglena has higher OM, CP and EE compared to grass hay and concentrate mixture (Table 1). Euglena contains 18 kinds of amino acids including all essential amino acids (Table 2). Saturated, mono unsaturated and poly unsaturated fatty acid content of Euglena are 64.5, 9.8 and 19.7 g/100g of the total fatty acid respectively (Table 3). The GE content of Euglena is lower than that of the substrate but with higher digestibility.

3.2. The effects of Euglena inclusion on in vitro NH_3-N and VFA concentration and the protozoa population

Ammonia N concentration generally increased with increasing concentrations of Euglena, but also exhibited quadratic (P=0.001) and cubic (P=0.024) effects. Total VFA concentration decreased significantly (linear P<0.001) when the substrate was totally replaced by Euglena (Table 3). Molar proportion of acetate increased (quadratic P=0.007; cubic P<0.001) and tended to increase linearly (P=0.057), whereas the proportion of propionate decreased linearly (P<0.001) and tended to decrease quadratically (P=0.068), while butyrate increased (linearly P=0.022; quadratic P=0.012; cubic P<0.001). The acetate to propionate ratio increased linearly (P<0.001) and quadratically (P=0.002) with increasing concentrations of Euglena. The pH increased linearly (P=0.001) and quadratically (P=0.009) while the ORP decreased linearly (P<0.001) and quadratically (P=0.003). There was a linear (P<0.001) and cubic (P=0.047) reduction in protozoa population as the concentrations of Euglena increased.

3.3. *The effects of Euglena on in vitro CH_4 emissions, DM and OM digestibility*

Methane emissions (ml/g DM) decreased at an increasing rate, generally with increasing concentration of Euglena but also exhibited quadratic (P<0.001) and cubic (P<0.001) effects (Table 4). *In vitro* DM digestibility was improved (linear, P=0.003; quadratic P=0.04; cubic P<0.001) by addition of Euglena. Similar trend was followed by IVOMD.

Table 2: Amino acid profile of Euglena

Amino acid type	% of Euglena	% of the total amino acid
Arginine	1.53	6.85
Lysine	1.59	7.11
Histidine	0.63	2.82
Phenylalanine	1.06	4.74
Tyrosine	0.8	3.58
Leucine	1.92	8.59
Isoleucine	0.93	4.16
Methionine	0.49	2.19
Valine	1.51	6.76
Alanine	1.79	8.01
Glycine	1.18	5.28
Proline	1.43	6.4
Glutamic acid	2.66	11.9
Serine	0.98	4.38
Threonine	1.14	5.1
Aspartic acid	1.95	8.72
Tryptophan	0.4	1.79
Cysteine	0.36	1.61

Table 3. Fatty acid profile of Euglena for *in vitro* study

Fatty acids	Lipid Numbers	% of the total fatty acid	% of the Euglena
Capric acid	C10:0	0.3	0.04
Lauric acid	C12:0	5.0	0.69
Tridecylic acid	C13:0	8.3	1.14
Myristic acid	C14:0	35.4	4.88
Pentadecylic acid	C15:0	2.9	0.4
Palmitic acid	C16:0	10.2	1.41
palmitoleic acid	C16:1	2.9	0.4
Margaric acid	C17:0	0.6	0.08
Heptadecenoic acid	C17:1	1.5	0.21
Stearic acid	C18:0	1.8	0.25
Oleic acid	C18:1	5.4	0.74
Linoleic Acid	18:2 (n-6)	2.0	0.28
Alpha-linolenic acid	18:3 (n-3)	1.1	0.15
Eicosadienoic acid	20:2 (n-6)	2.3	0.32
Eicosatrienoic acid	20:3 (n-3)	0.2	0.03
Dihomo-gamma-linolenic acid	20:3 (n-6)	3.8	0.52
Eicosatetraenoic acid	20:4 (n-3)	1.0	0.14
Arachidonic acid (AA)	20:4 (n-6)	4.5	0.62
Eicosapentaenoic acid (Timnodonic acid)	20:5 (n-3)	0.8	0.11
Adrenic acid	22:4 (n-6)	2.6	0.36
Clupanodonic acid	22:5 (n-3)	0.2	0.03
Osbond acid	22:5 (n-6)	1.2	0.17
others		6.0	0.83

Table 4: Effects of Euglena inclusion on VFA concentration, NH_3-N concentration and protozoa count after 24 h of incubation

	Euglena concentrations (g/kg DM)						SEM	Effect		
	0	50	100	200	400	1000		linear	quadratic	cubic
Volatile fatty acids (mol/100mol)										
Acetic	62.7^{bc}	63.6^{b}	63.3^{bc}	61.6^{c}	62.8^{bc}	65.8^{a}	0.049	0.057	0.007	<0.001
Propionic	26.5^{a}	25.2^{ab}	25.3^{ab}	23.6^{bc}	21.9^{cd}	20.2^{d}	0.082	<0.001	0.068	0.893
Butryric	9.03^{b}	9.36^{b}	9.57^{b}	11.9^{a}	11.9^{a}	9.61^{b}	0.050	0.022	0.012	<0.001
Valeric	1.8^{c}	1.87^{c}	1.85^{c}	2.95^{b}	3.46^{b}	4.4^{a}	0.027	<0.001	<0.001	0.245
A:P[1]	2.36^{c}	2.52^{c}	2.50^{c}	2.64^{cb}	2.87^{b}	3.25^{a}	0.011	<0.001	0.002	0.113
TVFA (mmol/l)[2]	41.5^{a}	35.2^{a}	40.1^{a}	30.4^{ab}	30.6^{ab}	19.4^{b}	0.814	<0.001	0.171	0.392
Protozoa(cell/ml*10^6)	2.50^{a}	2.13^{ab}	1.88^{b}	1.88^{b}	1.75^{bc}	1.38^{c}	0.013	<0.001	0.637	0.047
NH_3-N (mg/ml)[3]	15.9^{c}	17.0^{c}	17.5^{c}	33.3^{b}	60.3^{a}	66.6^{a}	0.428	<0.001	0.001	0.024
pH	7.09^{b}	7.04^{b}	7.18^{b}	7.13^{b}	7.39^{ab}	7.81^{a}	0.017	0.001	0.009	0.361
ORP (mV)[4]	-421^{a}	-427^{ab}	-430^{ab}	-442^{bc}	-456^{c}	-480^{d}	0.658	<0.001	0.003	0.430

[1]A: P = acetate to propionate ratio; [2]TVFA = total volatile fatty acid; [3]NH_3-N = ammonia N;
[4]ORP = oxidation reduction potential
[a-d]means within a raw with different superscripts differ($P<0.05$)

Table 5: Effects of Euglena inclusion on *in vitro* CH_4 emission, DM and OM digestibility

	Euglena concentrations (g/kg DM)						SEM	Effect		
	0	50	100	200	400	1000		linear	quadratic	cubic
Methane emission										
ml 24 h^{-1}	166^{a}	150^{b}	150^{b}	150^{b}	132^{c}	85.5^{d}	0.340	<0.001	<0.001	<0.001
ml/g DM	17.3^{a}	15.7^{b}	15.7^{b}	15.7^{b}	13.8^{c}	8.91^{d}	0.033	<0.001	<0.001	<0.001
ml/g digestible DM	24.9^{a}	20.9^{bc}	19.6^{cd}	21.7^{b}	18.9^{d}	9.02^{e}	0.059	<0.001	0.02	<0.001
ml/g OM	18.2^{a}	16.5^{b}	16.4^{b}	16.3^{b}	14.2^{c}	8.90^{d}	0.035	<0.001	<0.001	<0.001
ml/g digestible OM	29.9^{a}	24.6^{b}	22.8^{bc}	25.2^{b}	21.3^{c}	9.39^{d}	0.077	<0.001	0.024	<0.001
IVDMD[1]	0.70^{d}	0.75^{c}	0.80^{b}	0.72^{cd}	0.73^{c}	0.99^{a}	0.002	0.003	0.040	<0.001
IVOMD [2]	0.61^{d}	0.67^{c}	0.72^{b}	0.65^{cd}	0.67^{c}	0.95^{a}	0.002	0.001	0.022	<0.001

[1]IVDMD = *in vitro* dry matter digestibility; [2]IVOMD = *in vitro* organic matter digestibility;
[a-e]Means within a raw with different superscripts differ (P<0.05)

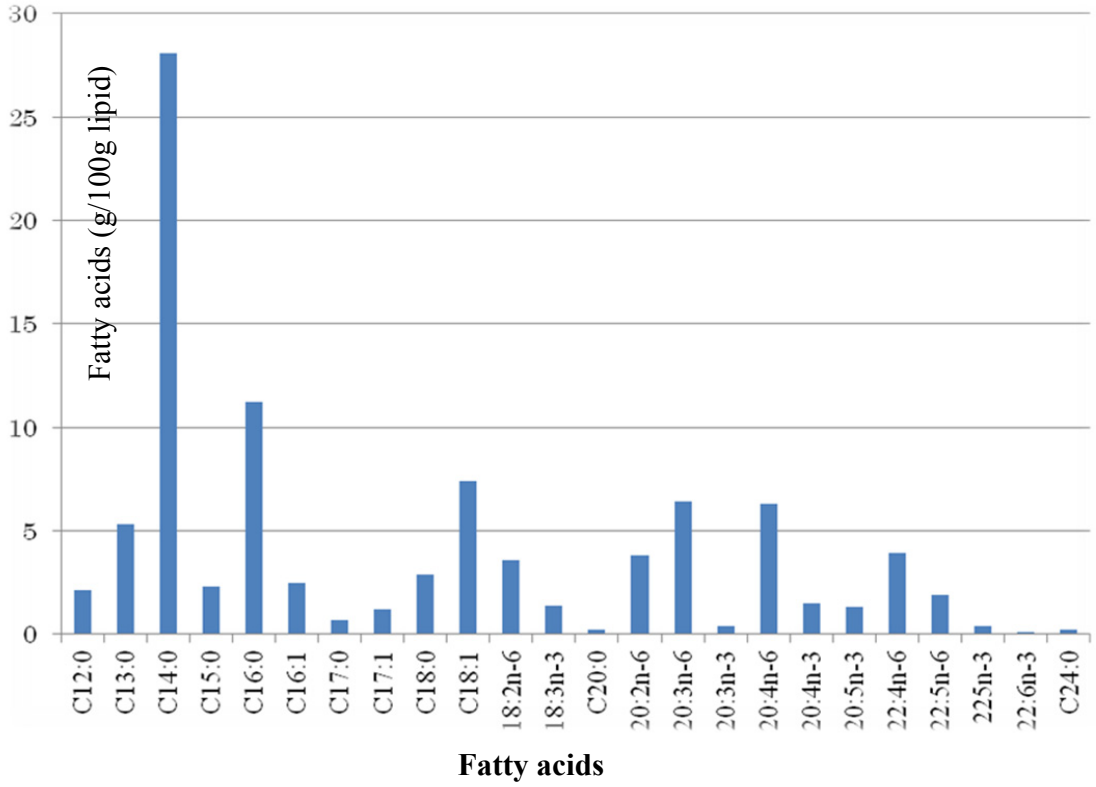

Fig 1. Fatty acid profile of Euglena

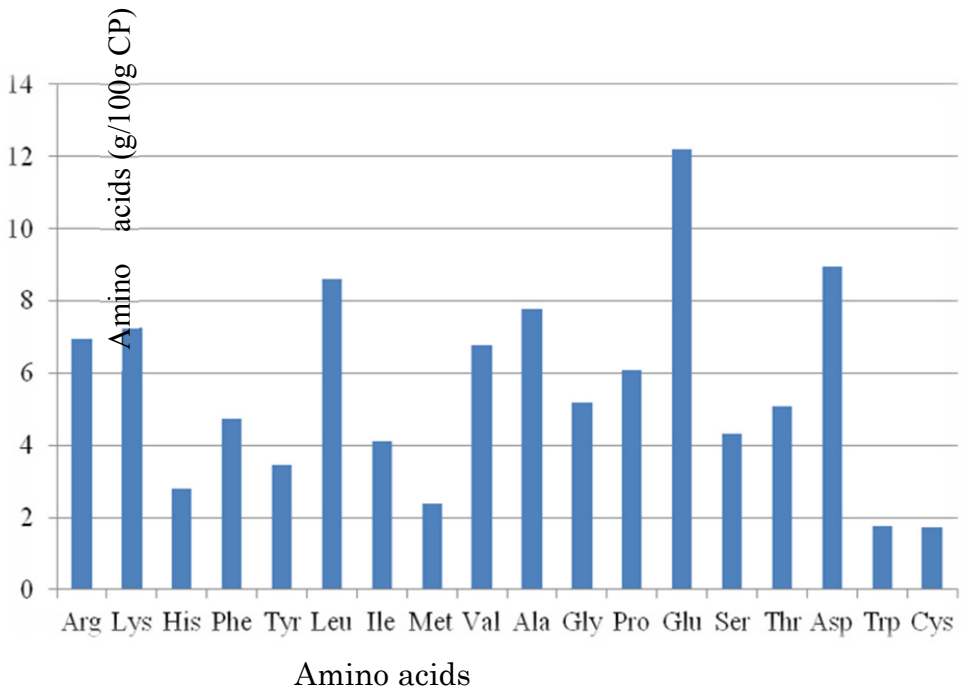

Fig 2. Amino acid profile of Euglena

4. Discussion

4.1. The effects of Euglena on in vitro CH_4 emission and digestibility

Methane emissions were reduced by 9 - 48%, when Euglena was included in a dose dependent manner. It has been reported that for every 1% addition of fat in the ration of ruminants, methanogenesis was reduced by 2.2 to 5.6% (Eugene et al., 2008; Beauchemin et al., 2008; Martine et al., 2010). The finding of our study confirms that addition of Euglena (100 g/kg DM) increased the fat content of the ration by 1% and reduced methane emission by 9.1%. Medium chain fatty acids (MCFA) such as lauric acid and myristic acid, identified as substances strongly reducing microorganisms participating in methanogenesis (Dohme et al., 1999). In our study, the lauric acid (C12:0), tridecylic aicd (C13:0), myristic acid (C14:0) and palmitic acid (C16:0) constitute 59.2 g/100 g of the total fatty acid in Euglena. The presence of these saturated medium chain fatty acids in higher proportion might be responsible for reduction of methane emission by influencing microorganism involved in the process of methanogenesis.

The findings of our *in vitro* study indicated that DM and OM digestibility was positively influenced by addition of Euglena. Inclusion of Euglena (100 g/kg DM) improved DM and OM digestibility by 15.26% and 18.21% respectively. The presence of balanced amino acid profile in Euglena might improved the efficiency of dietary protein utilization by facilitating the growth of microbial population and also increased efficiency of digestibility of fiber and starch. Study by Yan et al. (2012) indicated that pig supplemented with fermented algae led to a better balanced microflora in the intestine and higher nutrient digestibility. Evidences show that both essential and non essential amino acids play important role in regulating the intestinal microbiota and anti-oxidant response (Wu, 2009).

4.2. The effects of Euglena inclusion on in vitro *NH_3-N concentration, VFA concentration and protozoa population*

Ammonia N concentration was not influenced when Euglena was included 50-100 g/kg DM of the substrate but when the concentrations increased above 100 g/kg DM, NH_3-N concentration increased two to four fold compared to the control. This is associated to the increased concentration of CP in the ration as the proportion of Euglena increased. The result of a previous study indicated that the presence of excess dietary protein leads to ammonia formation (Place and Mitloehner, 2010), reflecting the loss of dietary nitrogen and causing environmental pollution.

Euglena supplementation at concentrations of 50-400 g/kg DM did not influence total VFA concentration ($P>0.05$) but reduced significantly when the substrate was totally replaced by Euglena. The proportion of propionate reduced by 11, 18 and 24% when the concentration of Euglena goes beyond 100 g/ kg DM, while the proportion of acetate increased by 5% at total substitution of the substrate with Euglena and butyric acid increased by 31-32%, when Euglena was included 200-400 g/kg DM of the substrate.

The protozoa population was also influenced by Euglena addition, showing a 14.8 to 44.8% reduction in a dose dependent manner. The decrease in CH_4 emission could be associated to the decrease in protozoa population influenced by the presence of higher proportion of saturated medium chain fatty acids (C12:0, C13:0, C14:0 and C16:0). Previous studies have shown that the addition of fatty acids in the ration of animals negatively affects not only the protozoa population (Szumacher-Stabel et al., 2004; Varadyova et al., 2007) but also affects methanogenic bacteria (Ipharraguerre and Clark, 2003; Szumacher-Stabel et al., 2004). In general supplementation of Euglena at the concentrations of up to 100 g/kg DM did not affect the NH_3-N concentration, VFA production, pH and ORP.

5. Conclusion

Euglena is rich source of protein (balanced amino acid profile) and fatty acids. Addition of Euglena reduced methane emission and improved DM and OM digestibility. On the other hand when the concentration of Euglena increases beyond 100 g/kg DM, it affects NH_3-N, protozoa population and VFA concentration. Thus the result of this *in vitro* study suggests that for optimum reduction of methane emission (9.1%) and considerable improvement in DM and OM (15 and 18%) digestibility, the inclusion of Euglena in the ration should not go beyond 100 g/kg DM.

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CHAPTER FOUR

The Effect of Euglena (*Euglena gracilis*) supplementation on nutrient intake, digestibility, nitrogen balance and rumen fermentation in Sheep

Abstract

This *in-vivo* study was conducted to evaluate the effect of supplementation with different concentration of Euglena (*Euglena gracilis*) on nutrient intake, digestibility, nitrogen balance and rumen fermentation. Four rumen cannulated Corriedale wethers sheep with an average body weight of 44.25±3.86 kg were arranged in a 4×4 Latin square design and fed a basal diet of Guinea grass (*Panicum maximum*) hay and concentrate mixture at the maintenance level with four different concentration of Euglena (0, 50, 100 and 150 g/kg DM intake). The experiment was conducted over 80 days in four 20 day periods that consisted of 14 days of acclimatization, 5 days of measurement and 1 more day for rumen liquor sample collection. The data were subjected to polynomial regression analysis. Dry matter (DM), organic matter (OM), acid detergent fibre (ADF) and gross energy (GE) intake increased linearly and quadratically ($P<0.05$) with increasing concentrations of Euglena. Similarly crude protein (CP) intake was increased linearly ($P<0.01$). Dry matter, OM, NDF, ADF and GE digestibility were not affected by supplementation of Euglena ($P>0.05$) while apparent CP digestibility increased linearly ($P< 0.01$). As a result, protein retention (g/d) was increased linearly ($P<0.01$) and quadratically ($P<0.05$) with increasing concentrations of Euglena. Ruminal NH_3-N concentration increased (linear, $P<0.01$) while ruminal protozoa population reduced linear and cubic ($P<0.01$) with increasing doses of Euglena. Euglena supplementation at different concentration did not change ($P<0.05$) the total volatile fatty acid (VFA) concentration and the molar proportions of acetate, propionate, butyrate and the acetate: propionate ratio. The finding of this study indicated that the addition of Euglena increased nutrient intake without affecting total tract

digestibility. It has been also demonstrated that addition of Euglena at higher level (150 g/kg DM) improved CP retention by 31%, which may be associated with increased CP intake and increased CP digestibility. However at higher level of supplementation, NH_3-N concentration and protozoa count were negatively affected.

1. Introduction

Algae contain complex bioactive compounds and these are gaining importance in emerging technologies with nutritional and environmental applications (Dubois et al., 2013). Microalgae contain a large percentage of oil, with the remaining parts consisting of large quantities of proteins, carbohydrates, and other nutrients (Spolaore et al., 2006). This makes the post-oil extraction residue attractive for use as animal feed. The use of microalgae in addition to its nutritional importance, it is a simple and inexpensive method for carbon dioxide (CO_2) management, which is currently an important global issue (Poti et al., 2015). Our previous *in vitro* study demonstrated that Euglena is rich source of amino acid and fatty acids, and the presence of higher proportion of saturated medium chain fatty acids in Euglena affected ruminal protozoa activity with subsequent impact on methane emissions (Aemiro et al., 2016). The nutritional composition of Euglena suggests that it can serve as a valuable replacement for good quality protein and energy supplement. Studies on nutritional and toxicological evaluations demonstrated the suitability of micro algae biomass as a valuable feed supplement or substitute for conventional protein sources such as soybean meal, fish meal, and rice bran (Becker, 2007). Previous studies indicated that lipid supplementation in the diet of ruminants is the most promising approach to increase the energy density and product quality (Fiorentini et al., 2015). However, the performance response and supplemental lipid composition is complex and differ according to the specific diet (Grainger et al., 2010). It has been also reported that there was a reduction in DM intake with animals fed diets with supplemental fat (such as palm oil, linseed oil) compared with that of animals fed diets without fat depending on its concentration (Fiorentini et al., 2014; Shingfield et al., 2010; Wanapat et al., 2011).

Limited *in vivo* studies are available on supplementation of microalgae in the ration of ruminants and the results are inconsistent. Enrichment in the poly unsaturated fatty acid was observed after supplementation of algae up to 94 g/d in the diet of ewe

(Papadoulos et al., 2002); supplementation of 9.35 and 43 g/kg DM microalgae directly through the rumen fistula reduced DM intake by 10 and 45% compared to the control (Boeckaert et al., 2008); supplementation of microalgae to heifers at the dose of 50 to 150 g/d did not affect DM intake (Axman et al., 2015); inclusion of micro algae suspension (10% of their body weight) in the diet of calves did not improve CP and ME intake but crude fiber digestibility was improved (Chowdhury et al., 1995). Micro algae, despite its importance as a source of valuable nutrients for animals and management of environmental safety its potential has not yet been fully exploited. In addition, research evaluating the effect of Euglena supplementation on the response of ruminants has not been conducted so far and the present study was conducted to evaluate the effect of Euglena supplementation on intake, digestibility, nitrogen balance and rumen fermentation in sheep.

2. Materials and Methods

2.1. Euglena (Euglena gracilis).

Euglena *(Euglena gracilis),* powder form with 100% purity, was obtained from Euglena Co. Ltd., Japan. The chemical composition of Euglena and the substrate (grass hay and concentrate mixture) are indicated in Table 1. The amino acid and fatty acid profile are indicated in Table 2.

2.2. Animals, diets and supplements

Four rumen fistulated Corriedale wether sheep with body weights of 44.25±3.86 kg were used in a 4×4 Latin square design. The wethers were kept in an individual metabolic cages and fed at maintenance-level (55 g DM/kg $BW^{0.75}$/ day) basal diet of Guinea grass (*Panicum maximum*) hay and concentrate mixture twice daily (08:30 and 16:30), and all had free access to clean drinking water and a mineral block. The mineral block consisted of Iron oxide, 1742 mg; Ferric oxide, 196 mg; Copper sulphate, 377 mg; Cobalt sulphate, 66

mg; 1235 mg; Zinc sulphate, 1046 mg; Manganese carbonate, 77 mg; Calcium iodate, 33 mg; Sodium selenite, 33 mg and Sodium chloride, 971 g per 1 kg mineral block.

Table 1. Chemical composition of experimental feeds and diets

Items[1]				Levels of Euglena (g/kg DM)				SEM	Contrast[2]		
	Guinea grass	Concentrate	Euglena	0	50	100	150		L	Q	C
DM(g/kg)	955	951	969	953	954	955	956	0.07	0.203	0.977	0.981
OM (g/kg DM)	915	928	964	921	922	923	925	0.06	0.058	0.897	0.900
Ash (g/kg DM)	84.7	71.7	35.9	79.5^{a}	77.7^{ab}	76.6^{ab}	75.2^{b}	0.02	0.001	0.749	0.749
CP (g/kg DM)	101	182	285	134	139	140	143	0.07	0.081	0.501	0.504
EE (g/kg DM)	21.1	36.3	132	27.2^{b}	32^{ab}	36.1^{ab}	40.4^{a}	0.08	0.001	0.91	0.916
GE (MJ/kg DM)	17.5	17.8	21.4	17.6	17.8	18.0	18.1	0.08	0.061	0.979	0.992
NDF (g/kg DM)	650	232	6.5	483	482	481	483	0.05	0.789	0.303	0.904
ADF (g/kg DM)	368	375	2.8	371	371	371	370	0.03	0.567	0.814	0.876
ADL (g/kg DM)	20.3	7.4	0.8	15.2	14.8	15.1	15.2	0.04	0.886	0.771	0.827

[1]DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; GE: gross energy; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL acid detergent lignin
[a-b]Means within a raw with different superscripts differ($P<0.05$)

[2] L = linear, Q = quadratic, C = cubic

The treatments were as follows: treatment 1, control (60% Guinea grass hay and 40% concentrate mixture); treatments 2, (60% Guinea grass hay, 35% concentrate mixture and 5% Euglena); treatment 3 (65% Guinea grass hay, 25% concentrate mixture and 10% Euglena) and treatment 4 (68% Guinea grass hay, 17% concentrate mixture and 15% Euglena) per kg DM of the total ration. Euglena powder was thoroughly mixed with concentrate mixture in each treatment to facilitate intake and to avoid preference. The rations were formulated in such a way that they are iso- nitrogenous and iso- caloric.

2.3. Experimental procedure

The experiment conducted for 80 days with each 20-day period consisting of 14 days of acclimatization followed by a 5-day digestion trial and the last 1 day for rumen liquor sample collection. Samples of the offered feed, refusal, faces and urine were collected and analyzed for nutrient content following standard procedures. Samples of the rumen liquor were collected at 0, 2, 4, 6, 8 and 24 h after the morning feeding and were stored at -20°C for NH_3-N and VFA analysis. Ruminal pH for each sampling time was measured immediately after the sample taken. Ruminal liquor samples were also stored for counting of protozoan population according to the procedure of Ogimoto and Imai (1981).

2.4. Analysis of volatile fatty acids

Total VFA and its components were determined with a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (ULBON HR-52, 0.53 mm ID × 30 m, 3.0 μm) using 2-ethyl-n-butyric acid as an internal standard; samples were prepared for analysis according to Sar et al. (2005).

2.5. Feces and urine collection and preparation

Feces and urine were collected for 5 days during each period, and the fecal samples from each treatment were thawed, bulked, mixed and sub-sampled. Sub-samples were dried at 60°C for 48 h in a forced-air oven and ground to pass through a 1-mm sieve for subsequent laboratory analysis. Urine was collected into buckets containing 100 ml of 100 ml/l (v/v) sulphuric acid to reduce the pH below 3.0 and to prevent bacterial degradation of N compounds. Approximately 50 ml/l of the urine sample was sub-sampled and stored at -20 °C until the nitrogen and GE analysis.

2.6. Chemical analysis

Experimental samples were analysed for DM by drying at 135 °C for 2 h (930.15), and OM, total ash (942.05) and ether extract (EE) (920.39) were determined according to the procedures of AOAC (1995). N was determined by the Kjeldahl method (984.13) (AOAC, 1995) using an electrical heating digester (FOSS Tecator^{TM} Digester, Tokyo, Japan) and an automatic distillation apparatus (FOSS Kjeltec^{TM} 2100, Tokyo, Japan), and crude protein (CP) was then calculated as the amount of N × 6.25. Neutral detergent fibre (NDF) was estimated without amylase and expressed inclusive of residual ash according to the method described by Van Soest et al. (1991), which was also used to determine acid detergent fibre (ADF) and lignin. ADF was expressed inclusive of ash, and lignin was determined by the solubilization of cellulose with sulphuric acid. The gross energy (GE) content of the samples was analyzed in a Shimadzu auto-calculating bomb calorimeter (CA-4AJ, Shimadzu Corporation, Japan), and the NH_3-N concentration was analyzed according to Conway and O'Malley (1942).

2.7. Amino acid and fatty acid analysis of Euglena

Amino acid and fatty acid profile of Euglena sample was analyzed by Japan Food Research Laboratories, Japan. The amino acid composition except for tryptophan was carried out by an automated amino acid analyzer (JLC-500/V, JEOL ltd. Japan; Column, LCR-6 with 4 mm x 120 mm ID, JEOL, Co. Ltd., Japan). Tryptophan was analyzed by high performance liquid chromatography (HPLC, LC-20AD, Shimadzu, Co. Ltd., Japan; Column, CAPCELL PAK C18 AQ, 4.6 mm ID x 250 mm, Shiseido Co. Ltd., Japan; detector, Flourospectro photometer, RF-20Axs, Shimadzu, Co. Ltd., Japan). Mobile phase consisted of perchloric acid and methanol (80:20). The flow rate was 0.7 ml/min and the fluorescence excitation was at 285 nm and 40 ^{o}C.

Fatty acid composition of Euglena was determined by Gas chromatography, GC-1700, Shimadzu Co. Ltd., Japan equipped with FID. The fatty acids were separated on 30 m x 0.25 mm ID, DB-23 capillary column. Helium was used as a carrier gas at a flow- rate of 1.5 ml/min with split less injection at 250 ^{o}C and the detector temperature was 250 ^{o}C.

2.8. Statistical analysis

Data obtained from the *in vivo* study were subjected to ANOVA in a 4 x 4 Latin square design using a polynomial regression analysis (REG procedure) available in SAS (2010) with the model: $Yij = \mu + Ti + eij$, where *Yij* is the dependent variable; μ is the overall mean; *Ti* is the fixed treatment effect; and *eij* is the residual. The experimental unit was the individual animal. Differences among the means were identified using Tukey's multiple comparisons, and effects were considered significant when $P<0.05$ while trends were discussed at $0.05<P<0.10$. The standard error of the means was determined using the least squares means procedure (lsmeans option) in SAS (2010).

3. Result

3.1 Chemical composition of experimental feeds

Lipid, CP and GE concentration of Euglena is higher than that of the concentrate mixture and guinea grass hay used in this study (Table 1). Euglena contain inconsiderable amount of fiber (< 0.7g/100g DM). In the present study Euglena contains all essential amino acids and 24 kinds of fatty acids respectively (Table 2 and 3).

3.2 Intake and digestibility

There were a linear, $P<0.01$ and quadratic, $P<0.01$ increase in DM and OM intake as the concentration of Euglena increased in the diet of sheep (Table 3). Crude protein intake (g/d) increased linearly ($P<0.01$) while NDF intake did not vary ($P<0.05$) between Euglena supplemented and un-supplemented groups but with in Euglena supplemented groups, supplementation with 150 g/kg DM had significantly ($P<0.01$) lower NDF intake compared to 50 and 100 g/kg DM supplemented groups. Gross energy intake (MJ/d) and ADF intake (g/d) increased linearly and quadratically ($P<0.01$) with increasing Euglena supplementation in the diet of sheep. Digestible energy intake increased quadratically ($P<0.05$) with increasing supplementation of Euglena. Dry matter, OM, NDF, ADF and GE digestibility were not influenced by supplementation of Euglena ($P>0.05$). Apparent CP digestibility increased linearly ($P< 0.01$).

3.3 Gross energy intake and loss

Energy was lost both with feces and urine. Fecal energy loss (MJ/d) increased significantly ($P<0.05$) with increasing Euglena supplementation. Similarly fecal energy as a proportion of GE intake was also affected quadratic ($P<0.05$) and cubic ($P<0.01$), whereas energy concentration (MJ/d) in urine was not affected by Euglena supplementation (Table 4).

3.4 Crude protein balance and urinary and fecal CP loss

Urinary nitrogen loss (g/d) increased linearly and quadratically ($P<0.01$) with increasing concentration of Euglena and also tended to increase cubically ($P=0.07$). Euglena supplementation had no significant effect on fecal nitrogen losses ($P>0.05$). However fecal CP as a proportion of total CP intake numerically reduced from 22 to 18%. CP retained (g/d) increased linearly ($P<0.01$) and quadratically ($P<0.05$) with increasing Euglena supplementation (Table 5).

3.5 *Effect on rumen fermentation*

Ruminal pH was increased (linear and cubic, $P<0.01$) with increasing concentration of Euglena. Treatment x time of sampling interaction for rumen pH was also influenced quadratically ($P=0.015$) as indicated in Figure 1. Ammonia-N concentration increased (linear, $P<0.01$) and tended to increase (cubic, $P=0.06$). Treatment x time of sampling interaction for ruminal NH_3-N concentration was also influenced linear and quadratic ($P<0.01$) and cubic ($P<0.05$) with increasing Euglena supplementation (Figure 2). Total VFA concentration and proportions of individual fatty acids were not affected ($P>0.05$) by supplementation of Euglena (Table 6). Treatment x time of sampling interaction of ruminal VFA concentration was affected in a linear, quadratic and cubic ($P<0.05$) manner as indicated in Figure 3.

Table 2. Amino acid profile of Euglena

Amino acid	AA proportion (g/100g Euglena)	AA% of CP (g/100g CP)
Arginine	1.97	6.94
Lysine	2.06	7.25
Histidine	0.79	2.78
Phenylalanine	1.34	4.72
Tyrosine	0.98	3.45
Leucine	2.44	8.59
Isoleucine	1.17	4.12
Methionine	0.68	2.39
Valine	1.92	6.76
Alanine	2.21	7.78
Glycine	1.47	5.18
Proline	1.73	6.09
Glutamic acid	3.45	12.15
Serine	1.22	4.30
Threonine	1.44	5.07
Aspartic acid	2.54	8.94
Tryptophane	0.5	1.76
Cysteine	0.49	1.73
Total AA	28.4	100

AA, amino acid; CP, crude protein

Table 3. Fatty acid profile of Euglena

Lipid type	FA (g/100g lipid)	FA (g/100g Euglena)
C12:0	2.1	0.29
C13:0	5.3	0.74
C14:0	28.1	3.91
C15:0	2.3	0.32
C16:0	11.2	1.56
C16:1	2.5	0.35
C17:0	0.7	0.10
C17:1	1.2	0.17
C18:0	2.9	0.40
C18:1	7.4	1.03
18:2n-6	3.6	0.50
18:3n-3	1.4	0.19
C20:0	0.2	0.03
20:2n-6	3.8	0.53
20:3n-6	6.4	0.89
20:3n-3	0.4	0.06
20:4n-6	6.3	0.88
20:4n-3	1.5	0.21
20:5n-3	1.3	0.18
22:4n-6	3.9	0.54
22:5n-6	1.9	0.26
225n-3	0.4	0.06
22:6n-3	0.1	0.01
C24:0	0.2	0.03
SFA	53	7.37
MUFA	11.1	1.54
PUFA	31	4.31

SFA, saturated fatty acids; MUFA, mono unsaturated fatty acids; PUFA, polyunsaturated fatty acids

Table 4. The effect of Euglena supplementation on intake and digestibility in sheep

Items[1]	Levels of Euglena (g/kg DM)				SEM	Contrasts[2]		
	T1	T2	T3	T4		L	Q	C
DM intake (g/d)	710^{b}	742^{a}	761^{a}	757^{a}	2.393	<0.001	0.002	0.652
DM digested (g/d)	521^{b}	541^{ab}	553^{ab}	564^{a}	3.685	0.001	0.61	0.833
DM digestibility	0.74	0.73	0.73	0.74	0.005	0.832	0.307	0.847
OM intake (g/d)	653^{c}	684^{b}	703^{a}	701^{ab}	2.592	<0.001	0.002	0.648
OM digested (g/d)	490^{b}	509^{ab}	520^{ab}	532^{a}	3.24	<0.001	0.66	0.764
OM digestibility	0.75	0.74	0.74	0.76	0.005	0.893	0.232	0.766
CP intake (g/d)	96.7^{c}	105^{b}	110^{ab}	114^{a}	0.767	<0.001	0.213	0.955
CP digested (g/d)	66^{c}	72^{bc}	78^{ab}	83^{a}	0.765	<0.001	0.663	0.913
CP digestibility	0.68^{b}	0.69^{ab}	0.71^{ab}	0.72^{a}	0.005	0.009	0.485	0.851
NDF intake (g/d)	344^{ab}	351^{a}	351^{a}	341^{b}	1.126	0.509	0.001	0.708
NDF digested (g/d)	238	237	231	231	2.034	0.170	0.930	0.578
NDF digestibility	0.70	0.67	0.66	0.67	0.006	0.157	0.189	0.634
ADF intake (g/d)	168^{b}	176^{a}	180^{a}	179^{a}	0.566	<0.001	0.002	0.652
ADF digested (g/d)	105	108	105	113	1.287	0.131	0.414	0.193
ADF digestibility	0.63	0.61	0.58	0.62	0.826	0.570	0.105	0.244

[1]DM: dry matter; OM: organic matter; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre

[a-d]Means within a raw with different superscripts differ($P<0.05$)

[2] L = linear, Q = quadratic, C = cubic

Table 5. The effect of Euglena supplementation on fecal and urinary energy losses in sheep

Items[1]	Levels of Euglena (g/kg DM)				SEM	Contrasts[2]		
	0	50	100	150		L	Q	C
GE intake(MJ/d)	12.5^{c}	13.2^{b}	13.7^{a}	13.8^{a}	0.043	<0.001	0.002	0.631
DE intake(MJ/d)	6.59^{b}	7.02^{ab}	6.8^{ab}	7.44^{a}	0.096	0.027	0.597	0.1
GE digestibility	0.53	0.53	0.5	0.54	0.013	0.937	0.254	0.114
Fecal energy loss(MJ/d)	5.92^{c}	6.18^{b}	6.91^{a}	6.31^{b}	0.044	0.011	0.002	<0.001
Fecal energy (% of GE intake)	47.4^{b}	46.8^{b}	50.5^{a}	45.9^{b}	0.359	0.86	0.026	<0.001
Fecal energy (% of tota l energy loss)	93.6	93.1	93.6	93.6	0.248	0.681	0.508	0.296
Urinary energy (MJ/d)	0.41	0.46	0.47	0.43	0.012	0.447	0.431	0.907
Urinary energy (% of GE intake)	3.27	3.49	3.45	3.15	0.127	0.619	0.13	0.996
Urinary energy (% of total energy loss	6.45	6.94	6.4	6.43	0.248	0.681	0.508	0.296
Total energy loss	6.33^{c}	6.64^{b}	7.38^{a}	6.74^{b}	0.045	0.012	0.001	<0.001
Total energy loss (% of GE intake)	50.6^{b}	50.3^{b}	53.9^{a}	49.0^{b}	0.345	0.797	0.013	<0.001

[1] GE: gross energy; DE: Digestible energy
[a-c]Means within a raw with different superscripts differ(P<0.05)

[2] L = linear, Q = quadratic, C = cubic

Table 6. Effect of Euglena supplementation on urinary and fecal CP losses in sheep

Items[1]	Levels of Euglena (g/kg DM)				SEM	Contrasts[2]		
	0	50	100	150		L	Q	C
Urinary CP								
g/d	38.4^{c}	44.5^{b}	51.2^{a}	46.5^{ab}	0.737	0.002	0.002	0.070
As % of total CP intake	39.8^{b}	42.7^{ab}	46.4^{a}	41.0^{ab}	0.77	0.368	0.015	0.154
As % of total CP excreted	64.9^{b}	67.3^{ab}	70.6^{a}	69.8^{a}	0.537	0.004	0.185	0.35
Fecal CP								
g/d	21.0	21.7	21.3	20.1	0.416	0.48	0.306	0.972
As % of total CP intake	21.7	20.8	19.3	17.7	0.435	0.006	0.726	0.958
As % of total CP excreted	35.4^{a}	32.7^{ab}	29.4^{b}	30.3^{b}	0.537	0.004	0.185	0.35
Total CP excreted								
g/d	59.4^{b}	66.2^{ab}	72.5^{a}	66.6^{a}	0.877	0.013	0.002	0.140
As % of CP intake	61.5^{ab}	63.4^{ab}	65.7^{a}	58.6^{b}	0.66	0.413	0.003	0.998
CP intake (g/d)	96.7^{c}	105^{b}	110^{ab}	114^{a}	0.767	<0.001	0.213	0.955
CP retained (g/d)	32.6^{b}	34.3^{b}	34.2^{b}	42.9^{a}	0.692	<0.001	0.017	0.069
CP retained as % of CP intake	33.8^{b}	30.8^{b}	31.2^{b}	37.9^{a}	0.39	0.055	<.0001	0.438

[1]CP: crude protein

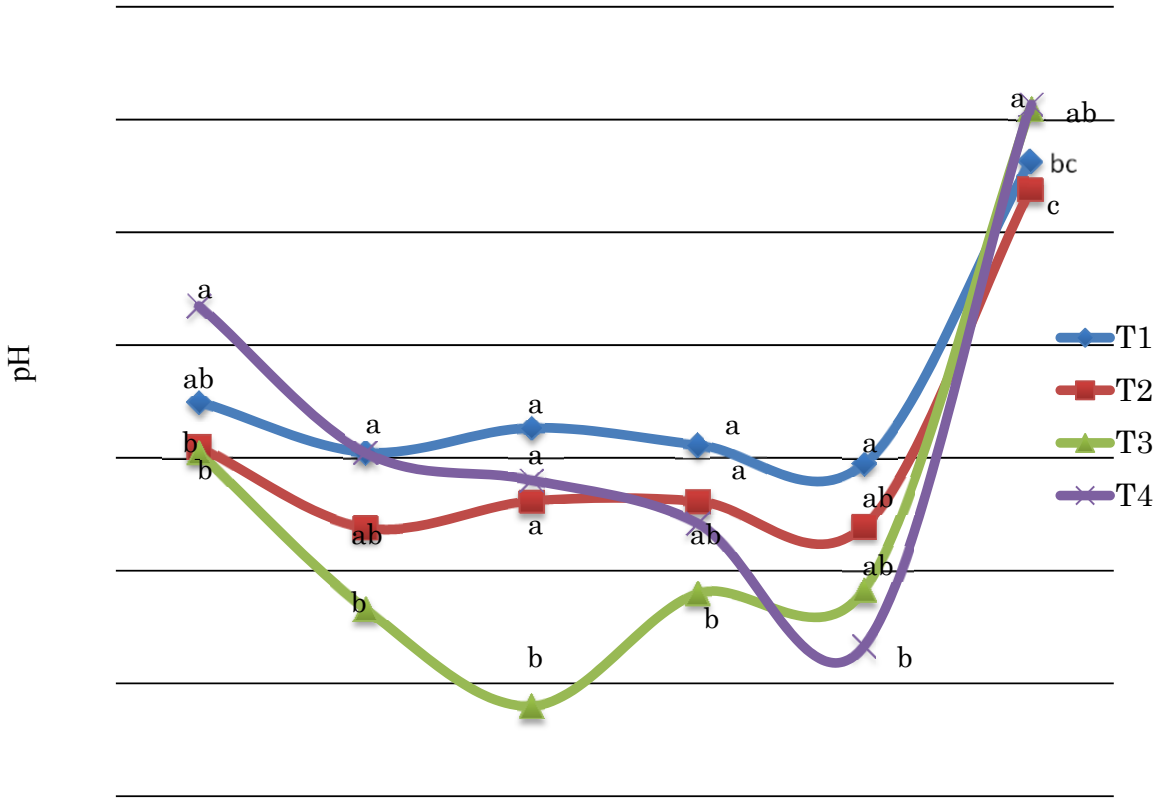
[a-c]Means within a raw with different superscripts differ($P<0.05$)

[2] L = linear, Q = quadratic, C = cubic

Table 7. Effect of Euglena supplementation on rumen fermentation and protozoa count for samples taken after 24 h of incubation

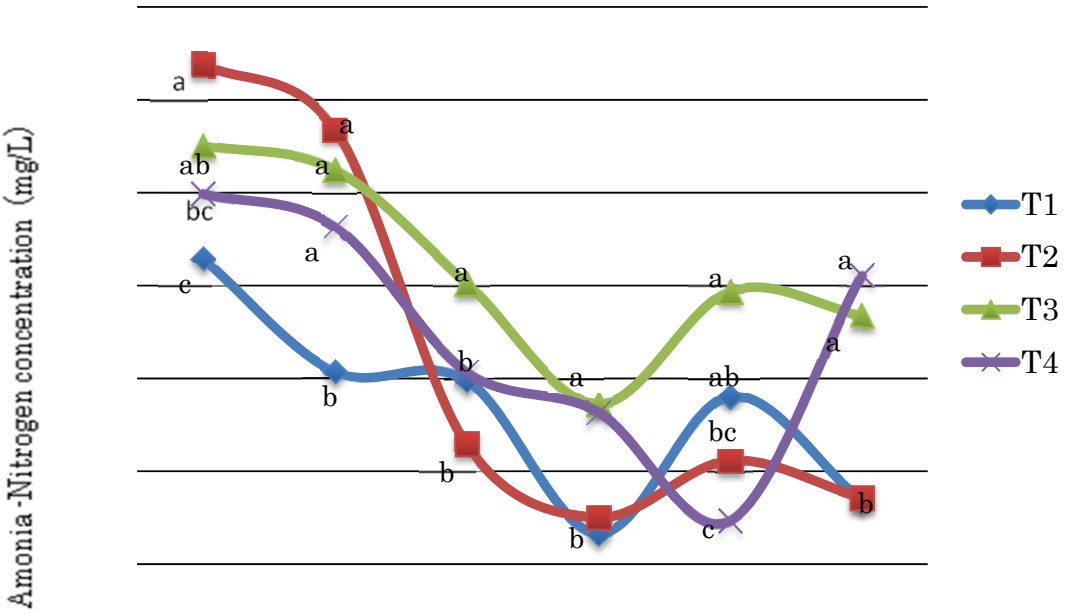
Items[1]	Levels of Euglena (g/kg DM)				SEM	Contrasts[2]		
	0	50	100	150		L	Q	C
Volatile fatty acids (mol/100mol)								
Acetate (A)	60.5	58.6	56.1	56.0	1.75	0.38	0.83	0.87
Propionate (P)	29.0	30.6	31.7	32.1	0.83	0.23	0.78	0.99
Butyrate	7.38	7.20	8.17	8.17	0.86	0.70	0.96	0.82
Valeric acid	2.95	3.23	3.70	3.37	0.15	0.27	0.38	0.59
Caproic acid	0.18	0.38	0.38	0.30	0.05	0.44	0.19	0.81
Total VFA (mmol/L)	48.3	47.9	48.4	50.6	2.09	0.73	0.79	0.98
A:P ratio	2.15	1.94	1.81	1.80	0.19	0.27	0.68	0.98
NH_3-N (mg/L)	41.8^{b}	42.8^{b}	61.8^{a}	66.2^{a}	1.82	<0.001	0.64	0.06
Protozoa (cell/l*10^6)	3.02^{a}	2.77^{b}	1.50^{c}	1.08^{d}	0.04	<0.001	0.34	<0.001
pH after 24 h	7.13^{bc}	7.08^{c}	7.22^{ab}	7.23^{a}	0.01	0.005	0.36	0.008

[1]VFA: volatile fatty acid; A:P: acetate to propionate ratio; NH_3-N, Ammonia N,
[a-d]Means within a raw with different superscripts differ($P<0.05$)
[2] L = linear, Q = quadratic, C = cubic



[a-b]Means within hour (column) with different superscripts differ(P<0.05)

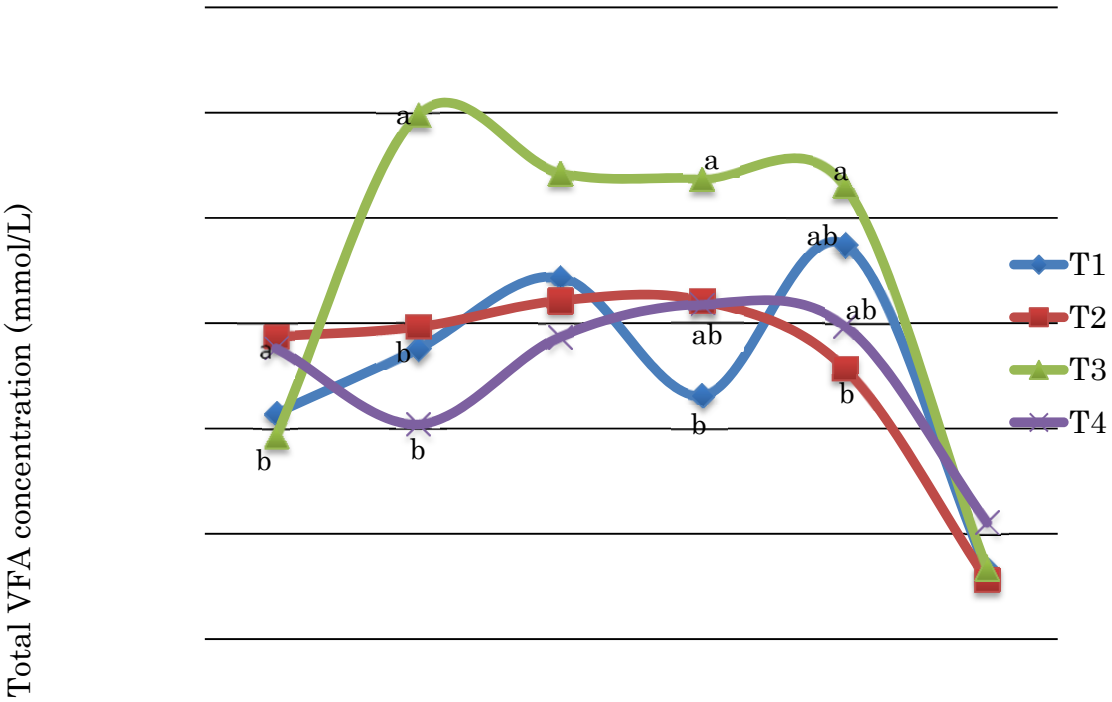
Figure 1. Effect of Euglena supplementation on ruminal pH in sheep, T1= 0 g/kg DM (control); T2= 50 g/kg DM; T3= 100 g/kg DM; T4= 150 g/kg DM



Sampling time after morning feeding, h

[a-c]Means within hour (column) with different superscripts differ($P<0.05$)

Figure 2. Effect of Euglena supplementation on ruminal NH_3-N concentration in sheep, T1= 0 g/kg DM (control); T2= 50 g/kg DM; T3= 100 g/kg DM; T4= 150 g/kg DM



[a-b]Means within hour (column) with different superscripts differ(P<0.05)

Figure 3. Effect of Euglena supplementation on VFA concentration, T1= 0 g/kg DM (control); T2= 50 g/kg DM; T3= 100 g/kg DM; T4= 150 g/kg DM。

4 Discussions

4.1. Effect of Euglena supplementation on nutrients intake

Euglena has an attractive nutrient profile and could serve as an alternative concentrate supplement in ruminants' diet. In the present study it has been observed that diets with Euglena (up to 150 g/kg DM intake) were readily consumed as noted by the linear increase in DM intake. Study by Axman et al., 2015 indicated that supplementation of microalgae to heifers at the concentration of 0, 50, 100 or 150 g/d did not affect DM intake but increased omega-3 fats concentration in blood plasma and hence improved yield grade. Another study indicated that algae meal is highly digestible by ruminants and readily consumed by lambs when included at up to 60% of the diet DM (Stokes et al., 2015). Dry matter and OM intake increased by up to 7.2 and 7.6% respectively when Euglena was included at a dose of 150 g/kg DM of the diet. On the other hand, study by Boeckaert et al., 2008 indicated that supplementation of micro algae (43 g/kg DM) reduced DM intake by 45% compared to the control. This might be associated with the nature of the fatty acid composition and the mode of application. They used fatty acids high in unsaturated fatty acid (USFA) and it was directly applied through the ruminal fistula. In the present study the medium chain saturated fatty acids (53 % of the total fatty acid) were the dominant lipid in Euglena and it was provided mixed with the concentrate part of the diet. Digestible DM and OM intake was also increased by 8.2 and 8.7% respectively at higher level of Euglena inclusion. Our data indicated that nutrient intake increased with increasing Euglena concentration. Crude protein intake increased by 9.2 to 21% with increasing concentration of Euglena (50-150 g/kg DM intake). This might be mainly associated with increased in dry matter intake facilitated by Euglena supplementation. Similarly digestible CP intake was also increased by 10.8-27.9%.

Neutral detergent fibre intake of the Euglena supplemented group did not vary

compared with the control group. However, with in the treated groups, NDF intake was lower at higher concentration of Euglena (150 g/kg DM). Digestible NDF intake was not influenced by addition of Euglena. Acid detergent fiber intake increased by 4.5-7.2%, while digestible ADF intake was not affected by addition of Euglena.

4.*2 Effect of Euglena supplementation on nutrient digestibility*

Ruminal digestibility is not impaired if diets contain fat less than 6% of the DM (Hess et al., 2008). In our study apparent DM digestibility remained unchanged and the coefficients of digestibility were 0.74, 0.73, 0.73, and 0.74 for 0, 50, 100 and 150 g/kg Euglena supplementation respectively. This shows that even though the total fatty acid concentration of the diet increased from 2.8% in the control group to 3.2-4.2% in the treated groups, digestibility was not affected. Organic matter digestibility was not also influenced by Euglena supplementation. Previous study (Castro et al., 2009) indicated that supplementation of vegetable oil (hydrogenated palm oil, 10.6 g/kg DM) in the diet of sheep (EE, 36 g/kg DM) increased apparent digestibility of OM and tended to increase that of DM but no significant difference in apparent digestibility of NDF and ADF were observed compared to un-supplemented group. Our data showed that the coefficient of apparent crude protein digestibility increased from 0.69 (control group) to 0.72 (150 g/kg DM supplemented group). This might be associated to higher digestibility of Euglena as indicated in our previous *in vitro* study (Aemiro et al., 2016). Gross energy, NDF and ADF digestibility was not influenced by Euglena and fatty acid concentrations in the diet. Previous study on the effect of fatty acids (oleic, linoleic and alfa-linolenic acid) supplementation at a dose of 35 g/kg w/w to a mixed diet containing 80% Lucerne and 20% barley did not affect degradation of DM, NDF and ADF (Jalc et al., 2007).

4.3 The effect of Euglena on rumen fermentation

It has been demonstrated that algae have the potential to assist rumen fermentation for improved gas production, and greenhouse gas abatement (Ubois et al., 2013). The present study showed that ruminal pH after 24-h of incubation increased from 7.13 to 7.23 with increasing Euglena concentration. Ruminal pH increased when micro algae was supplemented at a dose of 9.35 g/kg DM in the ration of dairy cows (Boeckaert et al., 2008). Similarly, previous study by Dubois et al., 2013 indicated that addition of algae increased post fermentation pH from 6.03 (control) to 6.06-6.33 (treatment group). Treatment x time of sampling interaction also affected ruminal pH. Among the different sampling times (0, 2, 4, 6, 8, 24), ruminal pH was highest at 24-h.

The concentration of NH_3-N in the rumen is a consequence of the balance between its production, absorption and utilization by microorganisms (Fiorentini et al., 2015). In this study ruminal NH_3-N concentration increased by 47.9 and 58.3% when Euglena was supplemented at the doses of 100 and 150 g/kg DM of the diet respectively. It is in agreement with our previous *in vitro* study, which stated that Euglena supplementation increased NH_3-N concentration by two to four fold when the concentrations of Euglena is above 100g/kg DM intake (Aemiro et al., 2016). This could be associated with higher nitrogen content and higher digestibility of Euglena. Improving the efficiency of microbial capture of released ammonia in the rumen by increasing carbohydrate availability is likely to reduce urinary nitrogen losses (Agle et al., 2010; Hristov et al., 2005). In our study nitrogen loss through urine was very small and it was not influenced by concentration of Euglena, which might be due to the presence of higher carbohydrate content in Euglena that facilitated the incorporation of NH_3-N to microbial protein. Otherwise ruminal ammonia nitrogen not utilized for microbial protein synthesis is likely to be excreted in urine, representing a net loss to the animal and contributing to environmental pollution (Tamminga, 1992). Treatment x time of sampling interaction at different incubation hours indicated that ruminal ammonia-N concentration was higher at early periods (zero and 2 h)

compared to higher incubation times.

The present data on the total ruminal VFA concentration after 24 hours of incubation indicated that it was not affected by Euglena supplementation. Our previous *in vitro* study also confirmed that Euglena inclusion by up to 400 g/kg DM did not affect total VFA concentration (Aemiro et. al., 2016). Molar proportions of acetate, propionate, butyrate and acetate: Propionate ratios were not also influenced by addition of Euglena. However at higher level of Euglena supplementation (150g/kg) though it is not significant, acetate reduced by 6.7%, propionate increased by 10.7% and acetate: propionate ratio decreased by 16.3% compared to the control. Treatment x time of sampling interaction indicated that higher concentration of ruminal VFA was obtained at 100 g/kg Euglena supplementation. Meta-analysis study by Patra, 2013 indicated that total VFA concentration and molar proportion of acetate were not affected by increasing concentration of fat in diets. Ruminal protozoa population reduced 8.3-64.2% with increasing doses of Euglena. It is in agreement with our previous *in vitro* study which stated that the protozoan population decreased by 15-45% with increasing Euglena concentration and this reduction may be linked to negative effects of saturated medium chain fatty acids present at higher proportion in the diet that affected microbial activity (Aemiro et al., 2016).

4.4. The effect of Euglena supplementation on energy intake and loss

Gross energy intake increased by 5.5-9.9% with increasing Euglena supplementation (50-150 g/kg DM intake). This might be associated with increase in DM intake influenced by Euglena supplementation. Similarly DE intake increased by up to 12.9% with Euglena inclusion at a dose of 150 g/kg DM. Findings of previous study indicated that inclusion of unicellular algae suspension (10% of their body weight) in the diet of calves did not improve CP and ME intake but crude fiber digestibility was improved (Chowdhury et al., 1995). Apparent gross energy digestibility was not influenced by Euglena supplementation and it was 0.53, 0.53, 0.50 and 0.54 of the GE intake respectively

for 0, 50, 100 and 150 g/kg Euglena supplementation. The majority of energy loss was through feces, which accounted 0.46-0.51 of the GE intake. Average daily fecal energy loss compared to GE intake was high (50.5 g/100g GE intake) at Euglena supplementation of 100 g/kg DM. However, when the Euglena concentration was increased to 150 g/kg DM, fecal energy loss was not affected compared to the control. This indicates that higher level of Euglena inclusion might have improved efficiency of energy utilization by facilitating efficiency of absorption in the lower digestive tract. In this study it was also observed that the overall urinary energy loss was very small (up to 3.5% of the total GE intake) and it was not influenced by Euglena supplementation.

4.5. *The effect of Euglena supplementation on CP intake and loss*

CP intake was 0.14 to 0.15 of the total DM intake and it increased with increasing concentration of Euglena. Total CP loss was 0.57 to 0.65 of the total CP intake indicating that the majority of the CP intake is lost with urine and feces. Urinary CP loss was 0.65-0.71 of the total CP loss and 0.40-0.46 of the total CP intake. In this study the data showed that urinary CP loss was higher at the diet with 10% Euglena supplementation (0.46 of the CP intake) but at highest level of supplementation (150 g/kg DM), urinary CP loss was not affected (41%) compared to the control group (40%). This shows that Euglena supplementation at higher level improved the efficiency of CP absorption in the lower tract. Fecal energy loss (g/d) remained unchanged among the treatment groups; however the fecal CP as the proportion of total CP intake reduced from 21.7% (control group) to 17.2% (highest supplementation group). The finding of this study showed that addition of Euglena reduced fecal CP loss and increased the efficiency of nitrogen availability and absorption in the lower tract. As a result, crude protein retention (g/d) increased by up to 31.4% when Euglena was supplemented at a dose of 150 g/kg DM compared to the control. Crude protein retained as a proportion of CP intake increased from 33.8% (control group) to 37.9% (highest Euglena supplemented group).

4.6. Amino Acid and Fatty acid profile of Euglena

The presence of balanced amino acid profile in Euglena enhances the efficiency of nutrient utilization and absorption in the lower tract in addition to facilitating ruminal fermentation for microbial growth and reproducibility. A continuous supply of essential amino acids plus sufficient nitrogen for synthesizing the other amino acids is essential for maintenance and production (Boisen et al., 2000). The proportion of lysine and methionine in Euglena (7.25:2.39) is in agreement with the proportion of ideal amino acid profile 6.7:2.0) shown by Boisen et al., 2000. Our study also indicated that Euglena is rich source of lipids, which contains 24 types of fatty acids. The total fatty acid consisted of 53% SFA and 42% USFA. Within the USFA, n-6 and n-3 content of Euglena are 25.9 and 5.1% of the total fatty acid contained in it. Previous study indicated that daily addition of 10 g dried algae (*Chlorella vulgaris*) in the diet of goats caused changes in the fatty acid profile of milk with concomitant increase in nutritional quality of goat's milk (Kourimska, et al., 2014). The presence of considerable amount of fatty acids help to control the activity of micro flora in the rumen and hence nutrients will be available in the lower digestive tract for enzymatic digestion which enhance the availability of diversified amino acids for absorption

Conclusion

The findings of this study indicated that the addition of Euglena increased DM, OM, and GE intake without affecting total tract apparent digestibility. It has been also demonstrated that addition of Euglena at a rate of 150 g/kg DM increased CP intake and CP digestibility with a concomitant increase in CP retention. However at higher level of supplementation, ruminal NH_3-N concentration and protozoa count were affected.

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General Summary

The inclusion of natural feed additives should be considered from the perspective of their effect on environmental safety and nutrient utilization efficiency. In this study natural feed additives (Sunphenon 30S-O and Euglena) were evaluated for their contribution towards reducing gas emissions and improving efficiency of nutrient utilization. Both *in vitro* and *in vivo* techniques were considered to evaluate the effect of these natural feed additives on gas emission, nutrient intake, digestibility and rumen fermentation parameters. The findings of the *in vivo* study indicated that Sunphenon 30S-O (20-40 g/kg DM) supplementation decreased CH_4 emissions (l/kg digestible OM intake) by 7.4-13.5% compared to the control, and our *in vitro* study similarly confirmed that supplementation (40-50 g/kg DM) reduced CH_4 emissions by 9.5 to 14.5% while CO_2 emissions declined by 6.4-13.8% compared to the control. The feeding experiment on sheep demonstrated that daily nutrient intake (DM, OM, CP, NDF or ADF) was not affected by supplementation with 10-25 g of Sunphenon 30S-O per kg of DM, but when the concentration was increased to 40 g/kg DM, the intake of DM, OM, NDF and ADF was reduced by 15.6% and that of CP by 13.9%. Conversely, the inclusion of Sunphenon 30S-O at different concentrations did not affect the total-tract digestibility of DM, OM, CP, NDF and ADF, and the overall CP digestibility coefficient was 0.80, 0.80, 0.79 and 0.79 for concentrations of 0, 10, 25 and 40 g/kg DM of Sunphenon 30S-O, respectively.

Supplementation of Sunphenon 30S-O reduced energy loss through urine from 17.4% to 11.2% of the GE intake. Similarly energy loss with CH_4 reduced from 7.3% to 6.2% of the GE intake. Because of this energy retention was not affected by the addition of different concentrations of Sunphenon 30S-O. In this study, protein intake was reduced by up to 0.16 of the total CP intake at the highest level of Sunphenon 30S-O supplementation (40 g/kg DM). Crude protein loss accounted for 0.46–0.57 of the total CP intake, and most of the loss (0.54–0.64 of the total CP loss) was through urine, which was reduced by

17-41% under supplementation. Despite the reduction in total CP intake, retained CP was not affected by addition of Sunphenon 30S-O, and this could be attributed to the effect of supplementation, which reduced CP loss through urine and increased CP absorption efficiency in the lower tract.

The present *in vitro* study indicated that the protozoa population was reduced by 2-19% and total VFA concentration was reduced by 8.6-15.9% when Sunphenon 30S-O was included at concentrations of 20-50 g/kg DM of the substrate. It was also observed that *in vitro* NH_3–N concentrations decreased with increasing concentrations of Sunphenon 30S-O and were 2 and 8 % lower at 40 and 50 g /kg DM of the substrate, respectively, compared to the control.

Euglena has an attractive nutrient profile and could serve as an alternative concentrate supplement in ruminants' diet. The findings of our third *in vitro* gas production study indicated that there was optimum reduction (9.1%) in CH_4 emission when Euglena was included at lower levels (5-10% of DM) in the ration without any negative effect on the rumen fermentation process. *In vitro* DM and OM digestibility was also positively influenced by inclusion of Euglena. Addition of Euglena at 5 and 10% of the substrate improved whole tract digestibility of DM and OM by 8.3-15.3% and 10.2-18.2% respectively. However when the level of Euglena was increased to 20 and 40 % of substrate, the response was lower ($P<0.05$) compared to 5 and 10% substitution. This is attributed to an increase in the level of fat concentration in the ration due to increased incorporation of Euglena, which influenced the activity of microbes involved in the fermentation process. Thus the data of our *in vitro* digestibility study suggests that inclusion of Euglena at lower levels is the best options for better response in terms of DM and OM digestibility. Protozoa count was influenced by addition of Euglena and there was a reduction of 14.8 to 44.8% when Euglena was included 5 to 100% of the substrate. In addition the data also showed that NH_3-N concentration, VFA production, pH and ORP were not influenced at lower levels of Euglena inclusion. Thus based on this *in vitro* study inclusion of Euglena at

5-10% could be promising option to be considered for proper rumen fermentation process.

The findings of the fourth experiment on sheep indicated that diets with Euglena (up to 150 g/kg DM intake) was readily consumed as noted by the linear increase in DM intake while DM digestibility remained unchanged. On the other hand apparent crude protein digestibility increased from 0.69 (control) to 0.72 (150 g/kg supplemented group). Gross energy, NDF and ADF digestibility was not influenced by Euglena and fatty acid concentrations in the diet. The data also showed that ruminal pH after 24-h of incubation increased from 7.13 to 7.23 with increasing Euglena concentration while ruminal NH_3-N concentration increased by 47.9 and 58.3% when Euglena was supplemented at the doses of 100 and 150 g/kg DM of the diet respectively. Ruminal protozoa population reduced 8.3-64.2% with increasing doses of Euglena.

The majority of energy loss was through feces, which accounted 46-51% of the GE intake. Fecal energy loss was not affected at higher level of supplementation. It was also observed that the overall urinary energy loss was very small (up to 3.5% of the total GE intake) and it was not influenced by Euglena concentration. Total CP loss through urine and feces was 0.57 to 0.65 of the total CP intake respectively and the data showed that urinary CP loss was higher at the diet with 10% Euglena supplementation (46.4% of the CP intake) but at highest level of supplementation (15%), urinary CP loss was not affected (41.0%) compared to the control group (39.8%). Hence CP retention (g/d) increased by up to 31.4% with Euglena supplementation of 150 g/kg DM compared to the control. This might be due to the contribution of Euglena supplementation towards improving efficiency of protein absorption in the lower tract.

In general this study confirmed that the incorporation of natural feed additives such as green tea extract (Sunphenon 30S-O) and Euglena in the ration of ruminants could help to improve efficiency of nutrient utilization and reduce CH_4 emissions with a concomitant reduction in energy loss.

General Conclusion

The major constituents of catechin present in Sunphenon 30S-O, which could be responsible for influencing CH_4 emission and other rumen fermentation parameters are Epigalo catechin gallate and Epigalo catechin. Both *in vitro* and *in vivo* studies confirmed that the addition of Sunphenon 30S-O reduced CH_4 emissions in a dose-dependent manner. Although supplementation reduced feed intake in sheep, the total-tract digestibility of nutrients was not affected regardless of the Sunphenon 30S-O concentrations in the diet. This study indicated that a relatively high concentration of Sunphenon 30S-O (50 g/kg of the substrate) exerted a negative effect on *in vitro* nutrient degradability, the protozoa population, NH_3-N and VFA concentrations. Thus, to achieve optimum CH_4 reduction and save dietary energy without any negative effect on whole-tract nutrient digestibility, Sunphenon 30S-O supplementation up to 40 g/kg DM of the diet could be a possible option.

Euglena with its higher nutritional value and digestibility, can substitute good quality protein supplement in the ration of ruminants. Our *in vitro* study indicated that addition of Euglena at lower levels has shown positive effect in CH_4 reduction and also improved DM and OM digestibility. At higher levels of Euglena inclusion, CH_4 reduction was more pronounced but VFA concentration and protozoa population were negatively affected. Thus, from *in vitro* study it can be concluded that addition of Euglena at 10% of the substrate is promising options to be considered for optimum reduction of CH_4 (9.1%) and considerable increase in digestibility of DM and OM (15 and 18%) respectively without any negative effect on rumen fermentation process. In addition to this the *in vivo* study indicated that the addition of Euglena increased DM, OM, and GE intake without affecting apparent digestibility. It has been also demonstrated that addition of Euglena at higher level (150 g/kg DM) increased CP intake and CP digestibility with a concomitant increase in CP retention.

ACKNOWLEDGEMENT

I would like to express my great appreciation to my major supervisor, Professor Takehiro Nishida, Department of Animal & Environmental Hygiene, Obihiro University of Agriculture & Veterinary Medicine, Japan who was abundantly helpful and offered invaluable assistance, support and guidance throughout the duration of my studies.

I would like to gratefully acknowledge Professor Kazutaka Umetsu, Department of Animal & Food Hygiene, Obihiro University of Agriculture & Veterinary Medicine, Japan for his useful suggestion and support during my study.

My special thanks should be given to Professor Masaaki Hanada, Department of Life Science and Agriculture, Obihiro University of Agriculture & Veterinary Medicine, Japan for his willingness to give his time so generously for technical advice and encouragement during my research study.

I would like to thank all the students under the supervision of Professor Takehiro Nishida in Animal and Food Hygiene, for their support during my research work. I would also like to convey my thanks to the Obihiro University of Agriculture and Veterinary Medicine for granting me three years scholarship for my PhD study.

My special thanks are extended to the Ethiopian Institute of Agricultural Research for providing me support and encouragement throughout my PhD study. I would like to express my heartfelt thanks to my friends for their support and wishes for the successful completion of my PhD study.

Finally I sincerely wishes to express my love and gratitude to my beloved wife, Seblewengel; my son, Biruh and my daughter, Rekik for their understanding & endless love, throughout the duration of my studies.

要旨

本研究は、消化管からのメタン産生、養分摂取、飼料消化率、養分出納、揮発性脂肪酸(VFA)濃度、アンモニア態窒素(NH_3-N)濃度、および原生動物数において天然素材からなる飼料添加物を羊の飼料に混合する効果を評価するために実施された。研究は、動物への飼養試験と人工培養法によって4つの実験を実施した。天然素材からなる飼料添加物は、Sunphenon 30S-Oとミドリムシとした。Sunphenon 30S-Oは緑茶の葉から得られ、そのカテキン含有量によって規格化されている(210g/kg乾物(DM))。

ユーグレナは、植物と動物の両方の性質を持つ単細胞生物であり、100%純粋な粉末を株式会社ユーグレナから得た。

最初の実験では、混合培養液からのガス発生量の測定と人工消化試験によって、種々の濃度のSunphenon 30-O添加効果を評価した。実験では、ギニアグラス乾草1kgに対して0.0、20、40、および50gのSunphenon 30S-Oを添加した。

2番目の実験は、4×4 ラテン方格法に従って4頭のコリデール種羊に、ギニアグラス乾草1kgに対して0.0、10、25、および40gのSunphenon 30S-Oを添加したものを給与した。

実験は、21日間を1処理区として84日の間実施された。1処理区においては、14日間の馴致期間と5日間の消化試験および2日間の呼吸試験を行った。これらのデータは、重回帰分析に用いられた。

Sunphenon 30S-Oの添加濃度が増大するに従って、乾物(DM)、有機物(OM)、粗タンパク質(CP)、および総エネルギー(GE)摂取量が二次的($P<0.05$)および直線的に($P<0.01$)減少した。添加濃度に関係なく、見かけの消化率に影響はなかった。

有機物摂取量1kgあたりのめん羊からのメタン発生量は、添加濃度が増加するに従って直線的に13.5%まで減少した。このことは、*in vitro*の試験結果と同様であった。

尿とメタンへのエネルギー損失は、Sunphenon 30S-Oの添加濃度が増大するに従って、総エネルギー摂取量の17.4%から11.2%まで、および7.3%から6.2%までそれぞれ減少した。

*in vitro*の実験結果から、Sunphenon 30S-Oの添加濃度が増大するに従って、VFA(mmol/L)、NH_3-N濃度(mg/ml)、および原生動物数は減少(一次的$P<0.01$;二次的$P<0.01$)した。

本研究により、濃縮タンニンの前駆物質であるカテキンは、羊からのメタン排出量を減らす効果があることが示された。

3番目の実験では、混合培養液からのガス発生量の測定、人工消化試験によって、種々の濃度のユーグレナ添加効果をメタン産生量、消化率、VFAおよびアンモニア濃度、プロトゾア数によって評価した。

粗飼料60%、濃厚飼料40%の比率で混合した飼料の乾物1kg当たりに0.0、50、100、200、400、および1000gのユーグレナを添加し、第一胃液と24時間混合培養した時のメタン産生および発酵性状、96時間培養したときの消失率について検討した。

メタン発生量(ml/g乾物)は、ユーグレナの添加濃度が増大するに従って一次、二次($P<0.001$)、三次($P<0.001$)的に減少した。アンモニア濃度(mg/ml)は、ユーグレナの添加濃度が増大するに従って一次($P<0.001$)、二次($P<0.001$)、三次($P=0.047$)的に増加した。

総VFA濃度(mmol/l)は、1000gのユーグレナ(100%基礎飼料と置換)を添加した場合、かなり減少した($P<0.001$) 。プロトゾア数は、ユーグレナの添加濃度が増大するに

従って一次(P<0.001)、三次(P=0.047)的に減少した。人工消化試験による消失率は、ユーグレナの添加濃度が増大するに従って一次(P=0.003)、二次(P=0.04)、三次(P<0.001)的に増加した。これらの結果から、飼料の乾物1kg当たりに100gのユーグレナを投与すると、メタン排出量が9.1%減り、乾物消化率を15.3%高めることが明らかとなった。しかし、ユーグレナの添加濃度が増大するに従ってさらにメタン発生量は減少するが、アンモニア濃度、VFA濃度およびプロトゾア数には悪影響を及ぼす。ユーグレナは、アミノ酸および脂肪酸を豊富に含有している。そのなかでも、飽和中鎖脂肪酸を豊富に含有しており、このことがルーメンプロトゾアの活性に影響を及ぼすことによって、結果的にメタン産生量にも影響を及ぼすものといえる。

4番目の実験は、めん羊を用いた飼養試験を実施した。4×4ラテン方格法に従って、平均体重44.3±3.9kgのルーメンフィステルを装着した4頭のコリデール種羊に、ギニアグラス乾草1kgに対して0、50、100、150gのユーグレナを添加したものを給与した。養分摂取量、消化率、窒素出納およびアンモニア濃度を測定した。

実験は、20日間を1処理区として80日の間実施された。1処理区においては、14日間の馴致期間と5日間の消化試験および1日間の第一胃液採取試験を行った。これらのデータは、重回帰分析に用いられた。

ユーグレナの添加濃度が増大するに従って、乾物(DM)、有機物(OM)、酸性デタージェント繊維(ADF)、および総エネルギー(GE)摂取量が一次的および二次的(P<0.05)に増加した。同様に、粗タンパク質摂取量は直線的に増加(P<0.01)した。DM、OM、NDF、ADF、およびGE消化率にはユーグレナ添加の影響はみられなかった(P>0.05)。ユーグレナの添加濃度が増大するに従って、ルーメン内アンモニア濃度は直的に増加し(P<0.01)、プロトゾア数は一次的および三次的(P<0.01)に減少した。みかけの粗タンパク質消化率は、直線的に増加(P<0.01)した。結果として、粗タン

パク質蓄積量(g/日)は、一次的(P<0.01)および二次的(P<0.05)に増加した。ユーグレナの添加は、総VFA濃度、酢酸、プロピオン酸、酪酸のモル比および酢酸：プロピオン酸比に影響を及ぼさなかった(P>0.05)。

以上の結果から、飼料中乾物1kg当たり40gまでのSunphenon添加は、飼料の利用性に悪影響を及ぼすことなく適度なメタンの減少と同時にエネルギー損失を減らす効果があるものといえる。同様に、高濃度のユーグレナ添加は、飼料の利用性に悪影響を及ぼすことなく養分摂取量および窒素蓄積量を増加させることが明らかになった。一般的に、Sunphenonおよびユーグレナのような天然素材からなる飼料添加物の反芻家畜への給与は、温室効果ガス発生量を減少させ、養分の利用性を向上させ、これらの環境保護効果によって持続可能な農業を営むことを可能とし、農家に潜在的な恩恵をもたらすものといえる。