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

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Abstract

This *in vivo* study was conducted to evaluate the effect of supplementation with different rates 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 rates 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 (P<0.001) and quadratically (P=0.002) with increasing concentrations of euglena. Similarly, crude protein (CP) intake was increased linearly (P<0.001). Dry matter, OM, NDF, ADF and GE digestibility were not affected by supplementation of euglena (P>0.11) while apparent CP digestibility increased linearly (P=0.009). As a result, protein retention (g/d) was increased linearly (P<0.001) and quadratically (P=0.017) with increasing concentrations of euglena. Ruminal NH_3-N concentration increased (linear, P<0.001) while ruminal protozoa population reduced linear, quadratic and cubic *(*P<0.008*)* with increasing doses of Euglena. Euglena supplementation at different concentration did not change (P>0.23) 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 a dose of 150 g/kg DM improved CP retention by 31%, which may be associated with increased CP digestibility and efficiency of utilization. Thus, euglena supplementation up to 150 g/kg DM of the diet could be a possible option for substitution of protein and energy sources.

Abbreviations

ADF: acid detergent fibre; ADL: acid detergent lignin; A: P, acetate to propionate ratio; CP: crude protein; DE: digestible energy; DM: dry matter; EE, ether extract; GE, gross energy; NDF: neutral detergent fibre; NH_3-N: ammonia-N; OM: organic matter; TVFAs: total volatile fatty acids; VFA: volatile fatty acid

Key words
Euglena, Intake, Digestibility, Sheep, Protein

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 management, which is currently an important global issue (Poti et al., 2015). Our earlier *in vitro* study demonstrated that euglena (*Euglena gracilis*) is a rich source of amino and fatty acids, with the ability to affect protozoa population and therefore methane emission (Aemiro et al., 2016). The composition of euglena suggests that it can serve as a source of high-quality protein and energy. Studies on nutritional and toxicological evaluations demonstrated the suitability of microalgae 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 animals fed diets without fat (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 polyunsaturated 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 microalgae 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). Microalgae, despite its importance as a source of valuable nutrients for animals and management of environmental safety, its potential have not been fully exploited yet. Most of the previous studies with microalgae indicated that they contain a variable amount of CP, fiber and minerals depending on the production method (controlled environment, marine water or others). In addition to this the mode of feeding of microalgae was quite different (directly through the rumen cannula, Boeckaert et al., 2008; mixed with the ration, Stokes et al., 2015; or in suspension form, Chowdhury et al., 1995) which influenced the rumen fermentation process differently. It has been also observed that in some studies, specific microalgae were used in pure

form (Aemiro et al., 2016) and in others in mixed or enriched form (Boeckaert et al., 2008). Because of these reasons, the results reported were inconsistent and incomparable. From our *in vitro* study (Aemiro et al., 2016), euglena supplementation up to 100 g/kg DM had shown better response in terms of rumen fermentation parameters, which was the basis to set the levels of euglena inclusion for our *in vivo* study and thus the objective of this work was to investigate the effect of increasing dose rates of Euglena in the diets of sheep on dry matter intake, *in vivo* digestibility, nitrogen balance and rumen fermentation.

2. Materials and Methods

2.1. Euglena (Euglena gracilis)

Euglena, powder form with 100% purity, was obtained from Euglena Co. Ltd., Japan. The chemical compositions of euglena, Guinea grass hay and concentrate mixture are indicated in Table 1.

2.2. Animals, diets and supplements

Four rumen fistulated Corriedale wether sheep with body weights of 44.25±3.86 kg and nine years old were used in a 4×4 Latin square design. The wethers were kept in an individual metabolic cages and fed a basal diet of Guinea grass (*Panicum maximum*) hay and concentrate mixture twice daily (08:30 and 16:30) at maintenance-level (55 g DM/kg $BW^{0.75}$/ day), and the euglena was offered only in the morning (8:30 AM) mixed with the concentrate mixture. Guinea grass hay was offered 15 minutes after Euglena was offered. The zero hours sample was taken just before provision of Guinea grass hay in the morning feeding. The 24 hours sample was taken just before the morning euglena feeding. All sheep 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; Zinc sulphate, 1235 mg; Magnesium, 1046 mg; Manganese carbonate, 77 mg; Calcium iodate, 33 mg; Sodium selenite, 33 mg and Sodium chloride, 971 g/kg mineral block. The concentrate mixture (corn meal 510 g/kg; oil extracted soy meal 240 g/kg; rice bran 210 g/kg; and molasses, calcium carbonate and salt mixture 40 g/kg) was obtained from Chubushiryo animal feed processing Co. Ltd, Japan. The CP, GE and fiber concentrations in euglena, concentrate mixture and Guinea grass hay used in this study were different and incomparable. As a result, there was a need to balance the nitrogen and energy concentrations among the dietary treatments. Thus, there was a slight adjustment in the proportion of ingredients such as guinea grass hay and concentrate mixture while the concentrations of euglena was set at 0, 50, 100 and 150 g/kg DM of the diet to assure the dietary treatments were isocaloric and isonitrogenous to meet the maintenance requirement for mature wether sheep. The dietary treatments were as follows: treatment 1, control (600 g/kg Guinea grass hay and 400 g/kg concentrate mixture); treatments 2, (600 g/kg Guinea grass hay, 350 g/kg concentrate mixture and 50 g/kg euglena); treatment 3 (650 g/kg Guinea grass hay, 250 g/kg concentrate mixture and 100 g/kg euglena) and treatment 4 (680 g/kg Guinea grass hay, 170 g/kg concentrate mixture and 150 g/kg 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 diet sequences were 0, 50, 100 and 150 g/kg DM for sheep 1; 50, 100, 150 and 0 g/kg DM for sheep 2; 100, 150, 0 and 50 g/kg DM for sheep 3 and 150, 0, 50 and 100 g/kg DM for sheep 4 with 14 days allowed between treatments to assure no carry-over effects of the

previous treatment.

2.3. Experimental procedure

The experiment was conducted for 80 days with each 20-days period consisting of 14-days of acclimatization followed by a 5-days digestion trial and the last 1 day for rumen liquor sample collection. Samples of the offered feed, refusal, feces 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 euglena 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. Rumen liquor samples were also stored for protozoa count according to the procedure of Ogimoto and Imai (1981). The data of protozoa count was converted into log 10 form and the results were adjusted to obtain normal value.

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 in 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 later laboratory analysis. Urine was collected into buckets containing 100 ml of 100 ml/l (v/v) sulfuric acid to reduce the pH below 3.0 and to prevent bacterial degradation of nitrogen 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. Analysis of chemical composition

Experimental samples were analyzed 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). Nitrogen was determined by the Kjeldahl method (984.13) (AOAC, 1995) using an electrical heating digester (FOSS Tecator™ Digester, Tokyo, Japan) and an automatic distillation apparatus (FOSS Kjeltec™ 2100, Tokyo, Japan), and crude protein (CP) was then calculated as the amount of N × 6.25. The concentrations of neutral detergent fibre (NDF), acid detergent fiber (ADF) and lignin (sa) were measured according to the method described by Van Soest et al. (1991) and the results were expressed with residual ash. The gross energy (GE) concentration 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 and fatty acid profile of Euglena sample were 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; a detector, Flourospectro photometer, RF-20Axs, Shimadzu, Co. Ltd., Japan). The 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

Normality of data was verified and outliers were tested using the REG procedure (SAS Institute Inc., Cary, NC, USA). Data obtained from the *in vivo* study (DM intake, nutrient intake, digestibility and protozoa count) were subjected to ANOVA in a 4 x 4 Latin square design using REG procedure (SAS 2010) with the model: $Yij = \mu + Ti + Ai + Pi + eij$, where, Yij is the dependent variable; μ is the overall mean; Ti is the fixed treatment effect; Ai is the random animal effect; Pi is the random period effect and eij is the residual. Dietary treatments were considered fixed whereas sheep and periods were random variables. Total number of observations for intake and digestibility were 320 (treatment (4) * animal (4) * period (4) * data collection days (5)) and for protozoa count 192 (treatment (4) * animal (4) * period (4) * data collection days (1) * replication (3)). Data of ruminal pH, NH_3-N and VFA were analyzed as 4 x 4 Latin square using repeated measures, where the effect of sampling time and the interaction between treatment and sampling time were included in the model. The total number of observations for pH, NH_3-N and VFA were 1152 (treatment (4) * animal (4) * period (4) * sampling frequency (6) * replication (3)). 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

Concentrations of DM, OM, CP, GE and fiber components were not different among dietary treatments (Table 1). Ether extract concentration increased linearly (P=0.001) from 27.2 g/kg DM for the control diet to 40.4 g/kg DM for Euglena (150 g/kg DM) supplemented group (Table 1). Total ash concentration of the diet reduced linearly (P=0.001) with increasing rates of Euglena (Table 1). Euglena has an inconsiderable amount of the fibre components (6.5 g/kg DM

NDF, 2.8 g/kg DM ADF and 0.8 g/kg DM Lignin) as indicated in table 1. In the present study, the total essential and nonessential amino acid concentrations in Euglena were 14.3 and 14.1 g/100 g DM respectively. The total long chain and medium chain fatty acid concentrations in euglena were 13.0 and 0.3 g/100 g DM respectively. The total saturated and unsaturated fatty acid concentrations in Euglena were 7.4 g/100 g DM (53 g/100 g total fatty acid in Euglena) and 5.9 g/100 g DM (42 g/100 g of the total fatty acid in Euglena) respectively. The poly and monounsaturated fatty acid concentrations in euglena were 4.3 and 1.6 g/100 g DM respectively.

3.2 Effect of euglena supplementation on intake and digestibility

There were a linear, $P<0.001$ and quadratic, $P=0.002$ increase in DM and OM intake as the rates of euglena increased in the diet of sheep (Table 2). Crude protein intake (g/d) increased linearly ($P<0.001$) while NDF intake did not vary between euglena supplemented and unsupplemented groups but within euglena supplemented groups, supplementation with 150 g/kg DM had significantly ($P=0.001$) lower NDF intake compared to 50 and 100 g/kg DM supplemented groups (Table 2). Acid detergent fiber intake (g/d) increased linearly and quadratically ($P<0.002$) with increasing euglena supplementation in the diet of sheep. Dry matter, OM, NDF and ADF digestibility were not influenced by supplementation of euglena ($P>0.11$) (Table 2). Apparent CP digestibility increased linearly ($P=0.009$).

3.3 Effect of euglena supplementation on energy intake and loss

Gross energy intake (MJ/d) increased linearly and quadratically ($P<0.002$), while GE digestibility was not influenced ($P=0.11$) with increasing rates of euglena in the diet of sheep (Table 3). Digestible energy intake increased quadratically ($P=0.027$) with increasing supplementation of euglena (Table 3). Energy was lost both with feces and urine. Fecal energy loss (MJ/d) increased significantly ($P<0.011$) with increasing euglena supplementation (Table 3). Similarly, fecal energy as a proportion of GE intake was also affected quadratic ($P<0.026$) and cubic ($P<0.001$), whereas energy concentration (MJ/d) in urine was not affected by euglena supplementation (Table 3).

3.4 Effect of euglena supplementation on CP balance and urinary and fecal CP loss

Urinary CP loss (g/d) increased linearly and quadratically ($P=0.002$) with increasing concentration of euglena and also tended to increase cubically ($P=0.07$) as shown in Table 4. Urinary CP loss (as a proportion of total CP intake) was not affected at a higher rate of euglena inclusion (150 g/kg DM). Fecal CP loss (as a proportion of total CP intake) reduced linearly ($P<0.001$) with increasing euglena supplementation (Table 4). Crude protein retained (g/d) increased linearly ($P<0.001$) and quadratically ($P=0.017$) with increasing euglena supplementation (Table 4).

3.5 *Effect of euglena supplementation on rumen fermentation*

Ruminal pH increased linearly ($P=0.005$) and cubic ($P=0.008$) with increasing

concentration of Euglena. Treatment x time of sampling interaction for rumen pH was also influenced quadratically (P<0.015) as indicated in Fig 1. Ammonia-N concentration increased (linear, P<0.001) 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=0.022) with increasing euglena supplementation (Fig 2). Total VFA concentration and proportions of individual fatty acids were not affected (P>0.19) by supplementation of euglena (Table 5). However, we observed large numerical difference (16%) in A:P ratio when euglena was supplemented at a dose of 150 g/kg DM compared to the control. Treatment x time of sampling interaction of ruminal VFA concentration was affected in a linear, quadratic and cubic (P<0.04) manner as indicated in Fig 3. Total protozoa count reduced significantly (P<0.008) with increasing rates of euglena (Table 5).

4 Discussions

In the present study, in order to ensure isonitrogenous and isocaloric nutrient among diets, adjustment was made in the proportion of ingredients such as the amount of Guinea grass hay (600, 600, 650, 680 g/kg DM) and concentrate mixture (400, 350, 250, 170 g/kg DM) due to euglena supplementation (0, 50, 100, 150 g/kg DM) respectively. This adjustment did not affect the concentration of NDF, ADF, CP and GE among the dietary treatments. However, there was a change in EE concentration (272, 320, 361, 404 g/kg DM) with increasing rates of euglena. These differences may affect the rumen metabolism and thus may hinder the attribution of some of the observed effect of euglena supplementation. The result indicated that even though the dietary treatments were not different in total CP concentration, changes were observed in CP digestibility and CP balance with increasing concentration of euglena which might be associated with the higher digestibility of euglena and increased efficiency of nutrient utilization.

4.1. The effect of euglena supplementation on DM, OM, CP and fiber intake

Euglena has an attractive nutrient profile and could serve as an alternative concentrate supplement in ruminant diets'. 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. Supplementation of algae residue to steers increased forage utilization by increasing members Firmicutes of the rumen microbes (Mc Cann et al., 2014). Increasing rumen degradable protein intake from microalgae supplementation in cattle consuming pasture low in protein, resulted in an increased in Mitchell grass hay intake (Panjaitan et al., 2014). When an algae byproduct was fed as a protein source to finishing lambs, there was no effect on DM intake when fed at up to 20% of the diet (Dib et al., 2012). The recent 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, the study by Boeckaert et al. (2008) indicated that supplementation of microalgae (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, fiber content and the mode of application (directly through the rumen cannula). In the present study, the medium chain saturated fatty acids were the dominant lipid in euglena. In another study, it has been indicated that addition of microalgae (up to 45% of the ration) reduced DM intake from 20.3 kg/d to 18.2 kg/d (Van Emon et al., 2015). Our findings 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 an increase in dry matter intake facilitated by euglena supplementation. The higher digestible energy and protein content in euglena facilitated the efficiency of nutrient utilization (Aemiro et al., 2016) and reduced the retention time of digesta DM, NDF and lignin in the rumen with greater changes due to microalgae supplementation (Panjaitan et al., 2014).

In this study, NDF intake of the euglena supplemented group did not vary compared to the control group. However, within the treated groups, NDF intake was lower at higher concentration of euglena (150 g/kg DM). Findings of the previous study by Van Emon et al. (2015) indicated that the NDF content of the ration increased as the proportion of the microalgae (15-45% of DM) increased in the ration of steers. This is due to the presence of high NDF content (340-370 g/kg DM), whereas in the present study euglena contained very little amount of NDF (6.5 g/kg DM). Because of the differences in the nutrient profile of microalgae used in different studies, comparisons among studies may not work.

4.2 *The effect of euglena supplementation on nutrient digestibility*

Rumen 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 EE concentration of the diet increased from 2.8% in the control group to 3.2 - 4.2% in the treated groups, apparent digestibility was not affected. Organic matter digestibility was not also influenced by euglena supplementation. The study by Drewery et al. (2014) indicated that when 100 mg N/kg BW post-extraction algal residue (Chlorella spp.) as a protein supplement was provided; organic matter digestibility of straw, low-quality forage, was maximized. Previous study by 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 unsupplemented 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 the higher digestibility of euglena as indicated in our previous *in vitro* study (Aemiro et al., 2016). Gross energy, NDF, and ADF digestibility were not influenced by euglena and fatty acid concentrations in the diet. Similarly, study by Jalc et al. (2007)

indicated that fatty acids (oleic, linoleic and alpha-linolenic acid) supplementation at a dose of 35 g/kg (w/w) to a mixed diet containing 80% Lucerne and 20% barley did not show any effect on DM, NDF and ADF degradation.

4.3 The effect of euglena supplementation on rumen fermentation

Feeding algae powder for growing wether goats (30 g/kg DM), increased rumen pH and reduced total VFA concentration (Zhu et al., 2016), which is in line with the general negative correlation between pH and total volatile fatty acids (TVFAs) concentrations (Huhtanen and Kukkonen, 1995). In the present study, post ruminal fermentation pH increased from 7.13 (control) to 7.22 – 7.23 (treatment group) when euglena was supplemented at doses of 100 and 150 g/kg DM respectively which indicates that pH increased in a dose-dependent manner. In agreement with our findings, the 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). Among the different sampling times (0, 2, 4, 6, 8, 24), ruminal pH was highest at 24-h. This might be associated with the decrease in VFA concentration as the time of rumen fluid sampling progressed from the morning feeding. The difference between 0 and 24-h observation was related to the difference in the reference point. The 0-h sampling of ruminal fluid was 15 minutes after euglena feeding, but before hay feeding, whereas the 24-h sampling was immediately prior to euglena feeding. Boeckaert et al. (2008) indicated that microalgae supplementation at a dose of 9.35 g/kg DM intake increased rumen pH, which was associated with decreased rumen short chain fatty acid concentrations. Hydrogenated fatty acid supplemented diets to steers increased ruminal pH and decreased the concentration of total VFA compared with the control diet, perhaps because the fat-supplemented diets contained less fermentable carbohydrate (Elliott et al., 1997). On the other hand, microalgae supplementation showed no effect on rumen pH measured at 6-h post feeding (Van Emon et al., 2015).

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 concentrations 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 was above 100g/kg DM intake (Aemiro et al., 2016). Improving the efficiency of microbial capture of released ammonia in the rumen by increasing carbohydrate availability is likely to reduce urinary N losses (Agle et al., 2010; Hristov et al., 2005). In the present study, the addition of euglena (150 g/kg DM) reduced nitrogen loss through feces while urinary nitrogen loss remained unchanged despite a higher concentration of NH_3-N, which might be associated with increased efficiency of nitrogen utilization in the lower digestive tract. On the other hand, previous studies indicated that ruminal NH_3-N not utilized for microbial protein synthesis is likely to be excreted in urine, representing a net loss of the animal and contributing to environmental pollution (Tamminga, 1992). Treatment x time of sampling interaction at different incubation hours indicated that the ruminal NH_3-N

concentration was higher during the earlier incubation period (0 and 2-h) compared to later incubation times.

The present data indicated that the total ruminal VFA concentration after 24-hour of incubation was not affected by euglena supplementation. Our previous *in vitro* study also confirmed that euglena inclusion of up to 400 g/kg DM, did not affect of total VFA concentration (Aemiro et al., 2016). 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. Molar proportions of acetate, propionate, butyrate and A:P ratio were not also influenced by an addition of euglena. However, at higher concentration of euglena supplementation (150 g/kg) though it was not significant, acetate reduced by 6.7%, propionate increased by 10.7% and A:P ratio decreased by 16.3% compared to the control. In agreement with our result, Fiorentini et al. (2015) reported similar numerical differences where supplemental lipid sources in the diets of ruminants did not show any effect on ruminal VFA and A:P ratio. Zhu et al. (2016) reported similar numerical differences (16%) as significantly different when algae were supplemented at a dose of 30 g/kg DM of the diet. The difference in responses may be related to the changes in the proportion of hay and concentrate supplement in the diets that hinder the effect of euglena supplementation on rumen fermentation. Treatment x time of sampling interaction indicated that the ruminal VFA concentration was lowest for rumen fluids sampled at 24-h from the morning feeding. The study by Boeckaert et al. (2008) indicated that ruminal short chain fatty acid concentrations decreased as the time of rumen sampling after morning feeding progressed following diurnal variations. Ruminal protozoa population reduced by up to 7.0% with increasing doses of Euglena. It is in agreement with our previous *in vitro* study which stated that the protozoan population decreased with increasing euglena concentration and this reduction may be linked to negative effects of saturated medium chain fatty acids present at higher proportions in the diet that affected microbial activity (Aemiro et al., 2016). The inclusion of fatty acids in the ration of ruminants reduced the protozoa population (Szumacher-Strabel et al., 2004; Varadyova et al., 2007). Major changes in the rumen bacterial community, including Butyrivibrio spp were observed in steers fed the 3% fish oil diet (Kim et al., 2008).

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

In the present study, GE intake increased by 5.5-9.9% with increasing euglena supplementation (50-150 g/kg DM intake). This might be associated with an increase in DM intake influenced by euglena supplementation. Recent findings by Gutierrez et al. (2016) indicated that application of low concentrations of high-oil algae biomass enhanced daily weight gain of feedlot cattle which might be due in part to an apparent increase in efficiency of energy utilization and in part to an increase in dry matter intake. Algae have been shown to contain chemical attractants (Mustafa et al., 1997; Jaime-Ceballos et al., 2007) that promote feed intake in aquatic species (Tierney and Atema, 1988). On the other hand, findings of previous studies indicated that inclusion of unicellular algae suspension (10% of their body weight) in the diet of

calves did not improve 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 major 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/100 g GE intake) at euglena supplementation of 100 g/kg DM. When Euglena was supplemented at the rate of 150 g/kg DM, fecal energy loss (as a proportion of GE intake) was not affected compared to the control. This indicates that higher concentration (150 g/kg DM) of euglena inclusion might have improved the efficiency of energy utilization. In this study, it was also observed that the overall urinary energy loss was very small and it was not influenced by euglena supplementation.

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

Previous studies indicated that inclusion of unicellular algae suspension (10% of their body weight) in the diet of calves did not improve CP intake (Chowdhury et al., 1995). However, in the present study addition of euglena increased CP intake with increasing rates of euglena. This might be associated with increased DM intake. It has been also reported that nitrogen excreted in feces and urine accounts for a high proportion of N intake, which may be more than 70% of the daily N consumption (Tamminga and Verstegen, 1996). In the present study CP excretion was 59% of the total CP intake when euglena was supplemented at higher concentration (150 g/kg DM) while CP loss as a proportion of DM intake was not influence compared to the control (0.59 vs 0.61) despite increased in CP intake. Even though there was no difference in total CP concentration among the diets, there were changes in both nitrogen digestibility and nitrogen balance. This shows that euglena supplementation at higher concentration (150 g/kg DM) improved the efficiency of CP utilization by reducing fecal CP loss and thus increased CP retention, which suggests that euglena could potentially be used to substitute a considerable amount of protein supplement in the diets of a ruminant.

Conclusion

The findings of this study indicated that the addition of euglena increased DM, OM, and GE intake without any negative effect on total tract apparent digestibility. It has been also demonstrated that addition of euglena at higher concentration (150 g/kg DM) increased CP intake and CP digestibility with a concomitant increase in CP retention. Thus, euglena supplementation up to 150g/kg DM of the diet could be a possible option for substitution of protein and energy sources.

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Table 1

Chemical composition of experimental feeds and diets

Items[1]	Diet ingredients (g/kg DM)			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	0.66	0.203	0.977	0.981
OM (g/kg DM)	915	928	964	921	922	923	925	0.60	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}	0.24	0.001	0.749	0.749
CP (g/kg DM)	101	182	285	134	139	140	143	0.71	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}	0.77	0.001	0.91	0.916
GE (MJ/kg DM)	17.5	17.8	21.4	17.6	17.8	18.0	18.1	0.75	0.061	0.979	0.992
NDF (g/kg DM)	650	232	6.5	483	472	481	483	0.54	0.789	0.303	0.904
ADF (g/kg DM)	368	37.5	2.8	236	234	249	257	0.25	0.567	0.814	0.876
Lignin (g/kg DM)	20.3	7.4	0.8	15.2	14.8	15.1	15.2	0.37	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

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

[2] L: linear; Q: quadratic; C: cubic

Table 2

The effect of Euglena supplementation on intake and digestibility in sheep

Items[1]	Levels of Euglena (g/kg DM)				SEM	Contrasts[2]		
	0	50	100	150		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 (coefficient)	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 (coefficient)	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)	66.0^{c}	72.0^{bc}	78.0^{ab}	83.0^{a}	0.765	<0.001	0.663	0.913
CP digestibility (coefficient)	0.69^{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 (coefficient)	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 (coefficient)	0.63	0.61	0.58	0.62	0.008	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 row with different superscripts differ (P<0.05)

[2] L: linear; Q: quadratic; C: cubic

Table 3

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}	6.80^{ab}	7.44^{a}	0.096	0.027	0.597	0.1
GE digestibility (coefficient)	0.53	0.53	0.50	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 as a proportion of GE intake	0.47^{b}	0.47^{b}	0.51^{a}	0.46^{b}	0.004	0.86	0.026	<0.001
Fecal energy as a proportion of total energy loss	0.94	0.93	0.94	0.94	0.003	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 as a proportion of GE intake	0.03	0.04	0.04	0.03	0.001	0.619	0.13	0.996
Urinary energy as a proportion of total energy loss	0.06	0.07	0.06	0.06	0.003	0.681	0.508	0.296
Total energy loss (MJ/d)	6.33^{c}	6.64^{b}	7.38^{a}	6.74^{b}	0.045	0.012	0.001	<0.001
Total energy loss as a proportion of GE intake	0.51^{b}	0.50^{b}	0.54^{a}	0.49^{b}	0.004	0.797	0.013	<0.001

[1] GE: gross energy; DE: digestible energy

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

[2] L: linear; Q: quadratic; C: cubic

Table 4

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 a proportion of total CP intake	0.40^{b}	0.43^{ab}	0.46^{a}	0.41^{ab}	0.008	0.368	0.015	0.154
As a proportion of total CP excreted	0.65^{b}	0.67^{ab}	0.71^{a}	0.70^{a}	0.005	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 a proportion of total CP intake	0.22^{a}	0.21^{ab}	0.19^{bc}	0.18^{c}	0.002	<0.001	0.526	0.923
As a proportion of total CP excreted	0.35^{a}	0.33^{ab}	0.29^{b}	0.30^{b}	0.005	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 a proportion of CP intake	0.61^{ab}	0.63^{ab}	0.66^{a}	0.59^{b}	0.007	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 a proportion of CP intake	0.34^{b}	0.31^{b}	0.31^{b}	0.38^{a}	0.004	0.055	<0.001	0.438

[1]CP: crude protein

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

[2] L: linear; Q: quadratic; C: cubic

Table 5

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	0.641	0.06
Protozoa count (LOG converted value)	6.48^{a}	6.44^{a}	6.18^{b}	6.03^{c}	0.009	<0.001	0.008	<0.001
pH after 24 h	7.13^{bc}	7.08^{c}	7.22^{ab}	7.23^{a}	0.013	0.005	0.356	0.008

[1]VFA: volatile fatty acid; A:P: acetate to propionate ratio; NH_3-N, Ammonia N,

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

[2] L: linear; Q: quadratic; C: cubic

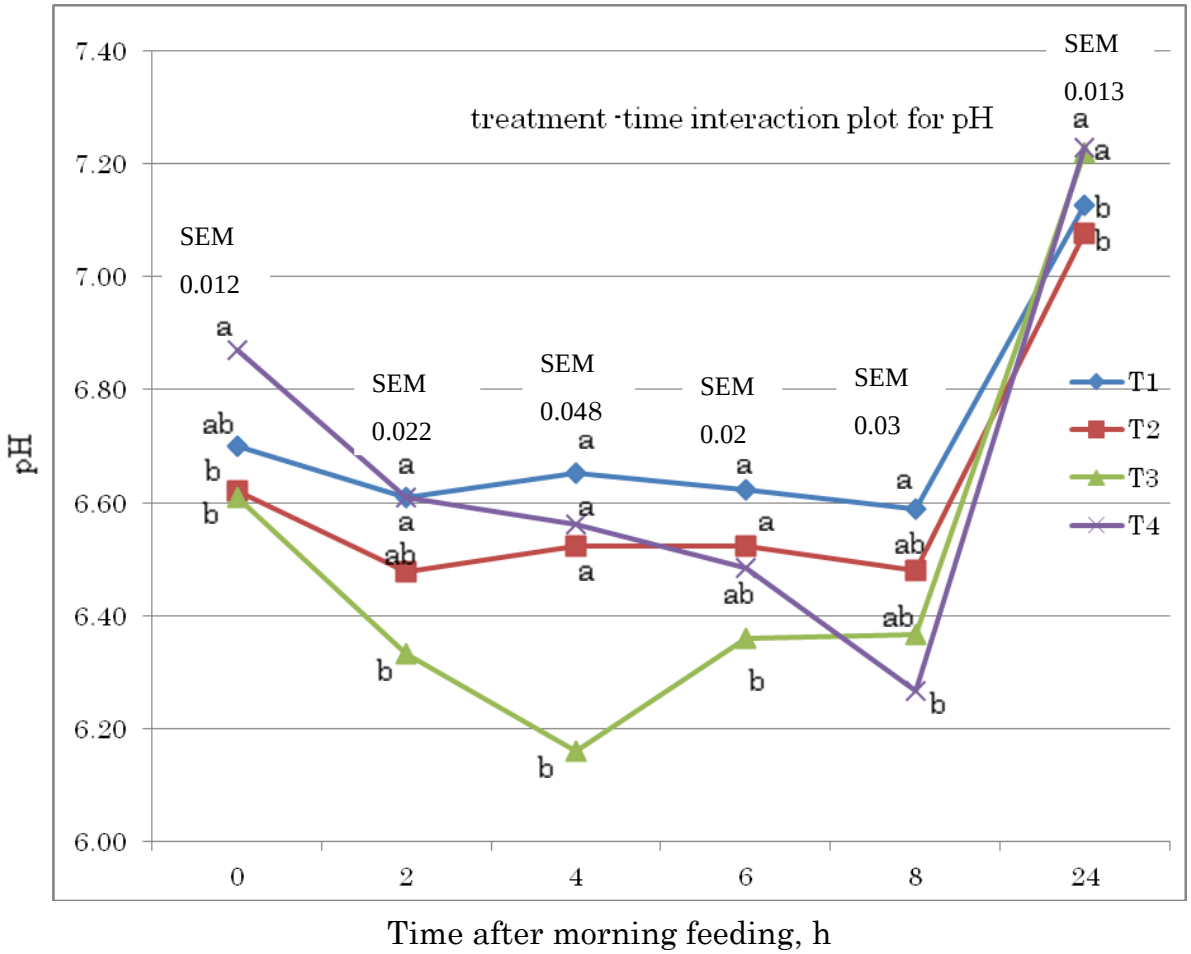

Figure 1. Effect of Euglena supplementation on ruminal pH in sheep, T1= 0 g/kg DM (control); T2= 50 g/kg DM; T3= 100 g/kg DM; T4= 150 g/kg DM, [a-b]treatment [x] time sampling interaction within hours with different superscripts differ ($P<0.05$), standard error mean (SEM) values of treatment x time sampling interaction for each hours are indicated along the sampling time after morning feeding.

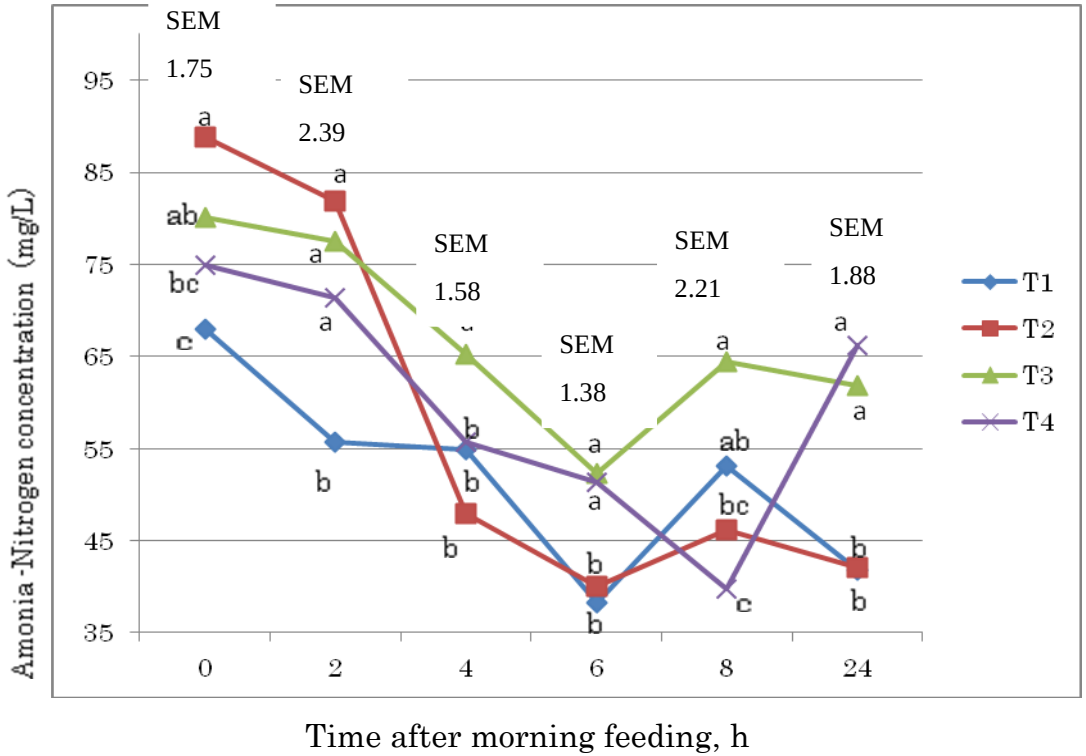

Figure 2. Effect of Euglena supplementation on ruminal NH_3-N (mg/L) concentration in sheep, T1= 0 g/kg DM (control); T2= 50 g/kg DM; T3= 100 g/kg DM; T4= 150 g/kg DM, $^{a\text{-}c}$treatment ^{x} time sampling interaction within hours with different superscripts differ (P<0.05), standard error mean (SEM) values of treatment x time sampling interaction for each hours are indicated along the sampling time after morning feeding.

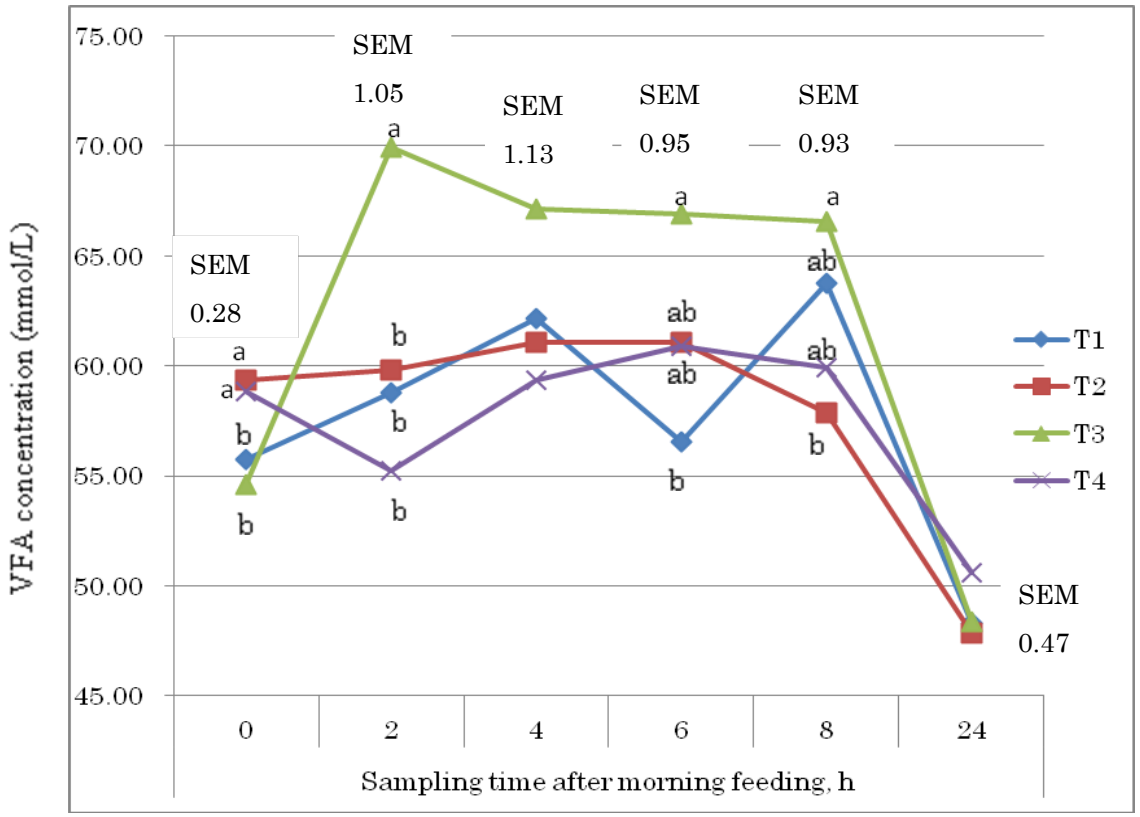

Figure 3. Effect of Euglena supplementation on VFA concentration, T1= 0 g/kg DM (control); T2= 50 g/kg DM; T3= 100 g/kg DM; T4= 150 g/kg DM, $^{a\text{-}b}$treatment ^{x} time sampling interaction within hours with different superscripts differ ($P<0.05$), standard error mean (SEM) values of treatment x time sampling interaction for each hours are indicated along the sampling time after morning feeding.