In Vivo and *In Vitro* Effects of Euglena Supplementation on Nutrients Intake, Digestibility, Rumen Fermentation and Enteric Methane Emission

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ウシ胃液培養液へのユーグレナの添加およびヒツジへのユーグレナ給与が栄養摂取量，消化率，第一胃内発酵性状およびメタン発生量に及ぼす影響

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DECLARATION

This is to certify that this is my original work and has not been presented for a degree or any other academic awards in any other university.

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DEDICATION

I humbly dedicate this work to my parents, Mr. and Mrs. Joseph Gathinji, my siblings, relatives, and friends for their earnest prayers during my post-graduate studies.

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Abbreviations and Acronyms

ADF:	Acid Detergent Fiber
AOAC:	Association of Official Analytical Chemists
CP:	Crude protein
DE:	Digestible Energy
DM:	Dry Matter
DMI:	Dry Matter Intake
EE:	Ether Extract
EO:	Essential Oils
FEL:	Fecal Energy Loss
GE:	Gross Energy
GHG:	Greenhouse gases
HAP:	Hyper-Ammonia Producing
IVDMD:	*In Vitro* Dry Matter Digestibility
IVOMD:	*In Vitro* Organic Matter Digestibility
KEPHIS:	Kenya Plant Health Inspectorate Service
ME:	Metabolizable Energy
MUFA:	Mono Unsaturated Fatty Acids
NDF:	Neutral Detergent Fiber
OM:	Organic Matter
ORP:	Oxidation Reduction Potential
PC:	Personal Computer
PUFA:	Poly Unsaturated Fatty Acids
RDP:	Rumen Degradable Protein
SEM:	Standard Error of the Means
SFA:	Saturated Fatty Acids
TEL:	Total Energy Loss
UEL:	Urinary Energy Loss

Definition of terms

Bioactive compound: These are constituents of Euglena that have effect on ruminal microbes.

Biohydrogenation: A process in which hydrogen is added into unsaturated fatty acids in the rumen by ruminal bacteria.

Digestibility: The proportion of nutrients in the feedstuff ingested by ruminant that is digested and absorbed (*in vivo*) or the extent to which the incubated feedstuff was fermented by rumen fluid microbes (*in vitro*).

Enteric methane: This is a gaseous by-product of fermentation process in a real or simulated rumen that is aided by symbiotic microbes that ferment the ingesta.

Experimental diet: This was a mixture of substrates into which the supplement was added to in order to evaluate its effect on various parameters.

Microalgae: These are microscopic eukaryotic and prokaryotic organisms that are typically found in marine or freshwater and grow by converting nutrients, carbon IV oxide, and sunlight into biomass (autotrophs) or utilize sugars (heterotrophs) or combine both modes of growth (Mixotrophs).

Ruminants: These are even-toed ungulates that have ability to obtain nutrients from plants by fermenting the ingesta in a four-chambered stomach by the help of symbiotic microbes before digestion in the lower digestive tract. They regurgitate the cud from rumen, the fore-chamber to mouth for mastication then re-swallow for further action by ruminal microbes, they include; the cattle, sheep, and goats.

Substrates: Feed resources onto which the ruminal microbes acted on.

Supplementation: Addition of a feed additive to a basal diet to enhance nutrients composition in order to exert desired effect.

Sustainability: The ability of a feed resource to meet the needs of the current generation without depletion, creating feed-food related conflict or compromising either the present or future generation meeting their needs as well.

Abstract

Background: The livestock sector is undergoing a paradigm shift in response to the burgeoning demand for food of animal origin. This is anticipated to have concomitant ramifications environmentally and in food sector. Environmentally, the increasing ruminant population will lead to a heightened accumulation of Greenhouse Gasses (GHGs) that results in global warming. Enteric methane emission represents nutrients utilization ineffectiveness by the ruminants since a substantial amount of energy is lost through the emitted gasses. Ingestion of low-quality feed resources by ruminants is becoming a pressing concern. There is a dire need to have high-quality feed supplements that can improve nutrients digestibility and abate enteric methane emission without compromising ruminants' productivity. Microalgae such as Euglena has been touted as supplements that can exactly do that and in a more sustainable manner due to their vast nutrients content, rapid production rate, and their ability to ensnare carbon dioxide during growth.

Objective: The prime objective of this study was to evaluate *in vivo* and *in vitro* effects of Euglena inclusion in the experimental diet on nutrients intake, digestibility, rumen fermentation parameters, and methane emission.

Methodology: The present study principally used a 4 × 4 Latin square design to evaluate the effects of Euglena inclusion in the experimental diet in two different directions; that is, the level of inclusion and the animal effect in the *in vivo* study. In the *in vitro* study, the same design was employed to evaluate whether the impact was exactly due to the level of inclusion or the fermentation jar effect. Both studies were conducted in quadruplicate to effect the methodological plan of the studies. Both studies utilized different Euglena to ensure reproducibility of the resultant data.

Results: All the data were subjected to a polynomial regression analysis of SAS 9.3 (*in vivo*) and 9.4 (*in vitro*). The results were broadly categorized into two due to the nature of experiments and their limitations. The broad categories are;
In vivo: All the nutrients intake were significantly influenced by Euglena supplementation ($p<0.05$). The supplementation only affected CP digestibility ($p<0.05$). Notably, the CP intake, the amount digested, and digestibility was significantly increased ($p<0.05$) while the CP lost in urine was significantly affected ($p<0.0001$) and the fecal CP was unaffected statistically ($p>0.05$). Energy metabolism was variedly affected, albeit the GE intake increased significantly ($p<0.0001$). The rumen fluid protozoal population plummeted significantly ($p<0.0001$) while NH_3-N concentration in the rumen fluid was significantly affected ($p<0.0001$). The supplementation did not exert any significant influence on pH and TVFA ($p>0.05$).
In vitro: Euglena inclusion in the diet numerically reduced CH_4 emitted by 35.4 percent, though the reduction was not statistically significant (p=0.142). TVFA and pH were unaffected statistically, protozoa were numerically (p= 0.161) reduced by 21.4 percent while NH_3-N concentration increased significantly (p= 0.0002). *In vitro* Dry Matter Digestibility (IVOMD) and in vitro Dry Matter Digestibility (IVDMD) were improved significantly ($p<0.05$).

Conclusion: Due to high protein and lipid content of Euglena, it significantly influenced nutrients intake, improved nutrient digestibility, and reduced CH_4 produced without compromising rumen fermentation significantly. Thus, Euglena can be an alternative ruminants' feed supplement to improve nutrients content of the diet, improve digestibility and abate enteric CH_4 in a sustainable way without compromising ruminants' productivity.

1 INTRODUCTION

1.1 BACKGROUND INFORMATION

Livestock is an indispensable global asset whose value is estimated to be about $1.4 trillion and directly supports livelihoods of over 600 million penurious peasant farmers in the developing countries (Thornton *et al.,* 2009). To underpin that livestock production is essential, livestock products alone contribute 33 percent of protein consumption and about 17 percent to the total kilocalories consumed globally as reported by Rosegrant *et al.,* (2009). Additionally, livestock plays other vital roles in the society such as being a source of income, drought power, soil conditioner and fertilizer and as an employment generator, especially in the developing world.

Livestock production especially ruminant production is experiencing a paradigm shift due to various factors that have prompted such a change. Ruminant production is burgeoning in response to the increasing demand for food of animal origin. There are various drivers that are triggering increment of demand for food of animal origin such as an unprecedented urbanization which is still set to continue in the next few decades, income increment especially in developing nations, and global human population which has been rising steadily and is still projected to hit 9-10 billion according to UNPD, (2008). In response to the increasing demand for food of animal origin, ruminant production has incredibly increased and for instance, beef production has doubled in the last few decades, coupled with carcass weight increment by about 30 percent according to FAO, (2010). Milk production per animal has increased by about 30 percent (FAO, 2010). Basing on the aforementioned animal products production increment and projected demand modifiers, it is undoubtedly clear that ruminant production will continue to increase markedly. Currently, the livestock production is said to occupy about 30 percent of the total ice-free terrestrial earth surface according to Steinfeld *et al.,* (2006). However, the percentage occupied is expected to change since the global cattle population is projected to almost double by 2050 from the current 1.5 billion to 2.6 billion while goats and sheep will increase by perhaps more than a billion from the current 1.7 billion to about 2.7 billion by the year 2050 (Rosegrant *et al.,* 2009).

Livestock production systems depict a disparate response to the increased demand for food of animal origin, due to their differences in the production processes and feed conversion efficiencies. For instance, poultry meat production system represents the quickest response to the increased demand for poultry meat due to the efficiency in feed utilization and short production cycle. On the other hand, beef, mutton, and goat meat production systems respond slowly to the burgeoning meat demand due to the slow reproduction cycle and low feed conversion efficacies as indicated by Sere and Steinfield, (1996). Ensuing the aforesaid ruminants low feed resource conversion efficiencies, scientists are gradually embarking on the pursuance of measures on how to bolster nutrients intake and digestibility. There are several strategies that have proved to be effective but some have been disputed and others banned due to their associated health implications in humans. Researchers are currently inclined toward the use of natural plant secondary metabolites and other related supplements which have been touted to be safer and environmentally friendly.

The increased ruminant production will have various effects but notable one will be extensive environmental effects. Environmentally, the increased ruminants production will have the following impacts as delineated by Delgado *et al,* (1999); expansion of grazing land and arable land for forage propagation which will trigger ramifications to the forest cover marked by unbridled deforestation, land degradation will result as most of the grazing land in arid and semi-arid areas are covered by unproductive-grass, eutrophication due to excessive leaching of nitrates to the groundwater and discharge of large amount of methane ensuing enteric fermentation. The latter sequel has lately become a cynosure in ruminants research, and scientists are trying the best way to mitigate enteric methane emission. A number of abatement strategies have been suggested, however, some of them have also been highly criticized due to their shortcomings. Scientists have a predilection for the most natural and sustainable ways of abating enteric methane emission, and environmentally benign methods to mitigate other negative environmental impacts. This has triggered a sustainable animal agriculture notion among the farmers and agricultural scientists due to the strain inflicted on natural resources as a result of increment of demand for food of animal origin. Stakeholders have been pondering continually on the conceptualization of the sustainable animal agriculture but WCED,(1987) defined sustainable agriculture development as one that meets the present needs without compromising the ability of future generation to meet theirs. Douglass, (1984) deduced from the aforementioned definition, that fruition of sustainable animal agriculture can only be achieved if resources intended to carry out a certain practice are in hand or are foreseeably available. Basing on the aforementioned concepts and reviews, animal nutritionists are gradually advocating for plant secondary metabolites, microalgae, and other related natural products whose merits in enhancing ruminant feed conversion efficiency and abatement of enteric methane emission outweigh risks involved if any.

Following the increment of needs for natural strategies to enhance nutrients conversion efficiency, nutrients supplementation, and enteric methane emission abatement, use of microalgae has gained traction in animal agricultural research. Microalgae have been touted as one of the most effective organisms in the conversion of ensnared carbon dioxide, sunlight, and inorganic nutrients into a biomass with vast nutrients (Richmond and Hu, 2013). There has been growing interest in exploring various microalgae use in various disciplines, animal feed being one of them. Microalgae have received consideration as a feed supplement to ruminants due to their high nutrients content, apparent ability to influence nutrients intake, digestibility, and possibilities to reduce enteric methane emission. However, the nutritive value of microalgae and the effects following inclusion in the ruminants' diet vary markedly depending on the species of microalgae, nutrients composition such as proteins, minerals, and oil contents and also on acclimatization of the animal to the supplemented diet (Harinder *et al.,* 2016).

1.2 PROBLEM STATEMENT

There has been growing concern about the ability to produce adequate animal products to suffice the projected demand increment for food of animal origin. Food demand is anticipated to escalate by up to 60 percent by the year 2050. The projected general food demand is expected to extend the strain to ruminants' production whose feed conversion efficiency is low. Ruminant production is rising and is expected to retain the same trend for the next couple of decades (Rosegrant *et al.,* 2009). The growth of ruminant population globally is expected to result into an environmental strain due to the expansion of feed propagation land, land degradation, and contribution to GHGs emission (Delgado *et al.,* 1999).

Enteric methane emission has numerous demerits, firstly, the gas contributes to global warming which has recently become a great concern. Secondly, it represents about 2-15 percent gross intake energy loss (Holter and Young, 1992). Johnson, (1995) ascribed the energy loss to the quantity and quality of the feed intake by ruminants. As a result, enteric methane emission represents one of the ineffectiveness in energy utilization by ruminants. Domesticated ruminants have been alleged to be the leading anthropogenic CH_4 emitters by 15 percent due to anaerobic enteric fermentation (Moss *et al.,* 2000).

The projected ruminant increment in response to the rising demand for food of animal origin is expected to be strenuous to the environment in relation to land degradation as a result of overstocking. Similarly, the expansion of forage propagation land will lead to forest encroachment which is perilous to water sources and biodiversity at large. Additionally, as of now, there are limited conventional feedstuffs such as soybean and corn which are the source of dietary energy and protein for ruminant feed. The aforementioned two crops are humans' staple food crop and hence food crisis and insecurity may arise in future if exploration of alternative sources of food/ feed supplement is not undertaken (Lum *et al.,* 2013).

Ruminants have been shown to have relatively low feed conversion efficiency as compared to other systems of animal production such as poultry production (Sere and Steinfield, 1996). This low feed conversion efficiency has been thought to be due ingestion of low-quality feed which has been largely associated with increased enteric methane emission which in turn leads to gross intake energy loss. Nutrients deficiencies have also been pointed out as an issue of concern in ruminant nutrition especially in the developing nations, supplementation remains an ideal solution to this hurdle.

Finally, nitrogen imbalance and loss in the environment has spurred a great concern. Excreted nitrogen via urine or feces has led to eutrophication after being leached into the groundwater. Ruminants both small and large ruminants have been shown to be among the leading animals in nitrogen excretion (Zervas and Tsiplakou, 2012). The highest global Nitrous oxide (N_2O) emitters from manure management are cattle leading by 60 percent, small ruminants by 19 percent and lastly monogastric by about 21 percent (See table 4; Lum *et al.,* 2013 & Zervas and Tsiplakou, 2012).

1.3 RESEARCH JUSTIFICATION

The anticipated ruminant production increment, coupled with the involvement of conventional feedstock for biofuel production, have spurred burgeoning of their prices. Due to the increment of food prices and crisis, food insecurity might be in the offing (Lum *et al.,* 2013). Apparently, this indicates that the use of conventional soybean and corn as protein and energy source in feed/food and recently in biofuels production is unsustainable. However, Microalgae such as Euglena have been perceived to have glaring properties for biofuel production and defatted biomass have been shown to be a promising carbon-neutral animal feed supplement or even to replace some corn and soybean without affecting the performance of livestock such as poultry (Austic *et al.,* 2013). Limited studies have been conducted to evaluate the effects of Euglena inclusion in the ruminant diet. The study thought is was reasonable to delve into the possibilities of including Euglena in the ruminants diet as a supplement.

Microalgae have been touted as a new source of nutrient supplement in animal diet due to their vast nutrients composition. Although microalgae's nutritional content varies considerably depending on the species of concern, most have been characterized by high protein, carbohydrates and lipid content somewhat higher than conventional feedstock (table 1; Lum *et al.,* 2013). Additionally, almost all microalgae have been shown to possess oodles of vitamins, fatty acids and amino acids especially the first limiting amino acid, lysine. Euglena Co. Ltd, a Tokyo-based company has explicitly illustrated that Euglena has over fifty-nine nutrients which can be of great value as supplements both to human and animal feeds. These reasons underpinned the necessity of this research to evaluate the effects of Euglena inclusion in the animal diet.

Among the several microalgae, *Euglena gracilis* has been shown to contain high amino acids and other nutrients content and thus implying that it has higher nutrition qualities compared to other microalgae as demonstrated by Nakano *et al.,* (1995). *In vitro* studies have suggested that Euglena inclusion in the animal diet may have an influence on dry matter digestibility and hence consideration of Euglena as an effective ruminant diet supplement is plausible.

With the recent and expected increment of GHGs in the environment, reliable and sustainable solutions are direly needed to mitigate this dicey environmental concern. Carbon dioxide (CO_2) concentration in the atmosphere has been increasing from various emission sources; hence, carbon-fixation systems are greatly needed. Use of microalgae for fixation have been shown to be the most effective and economical way of mitigating CO_2 in the atmosphere (Chae *et al.,* 2006). Euglena is known to grow under high concentration of CO_2, a thousand times more than normal air content. On the other hand, Euglena is highly believed that it can influence rumen fermentation and methane (CH_4) production due to the high fatty acid and protein content.

The research was highly persuaded that there was a necessity to evaluate all the intriguing attributes associated with Euglena as a feed supplement and investigate various effects ensuing Euglena inclusion in the animal diet.

1.4 Study hypothesis

There are no *in vitro* and *in vivo* effects of Euglena supplementation on nutrients intake, digestibility, rumen fermentation, and enteric methane emission.

1.5 Study objectives

1.5.1 General objectives

To evaluate *in vitro* and *in vivo* effects of Euglena supplementation on nutrients intake, digestibility, rumen fermentation, and enteric methane emission.

1.5.2 Specific objectives

I. To investigate the effects of Euglena supplementation on nutrients intake
II. To evaluate the effects of Euglena supplementation on nutrients digestibility
III. To examine the impacts of Euglena supplementation on rumen fermentation parameters
IV. To evaluate the impacts of Euglena supplementation on methane emission in an *in vitro* setup.

2 LITERATURE REVIEW

2.1 Ruminants' production

Ruminants' production constitutes a very vital component of livestock production and in the agricultural economy especially in the developing nations. This essential contribution extends beyond direct food production to include multipurpose dependency by the citizens of the said countries. The benefits accrued from ruminants production include; employment creation, the source of fertilizer, power, and fuels, the source of hides and skin and close linkage to social and cultural fetes (FAO, 1992). Ruminants include cattle, sheep, goats, deer, giraffes and buffaloes. The former three forms valuable and domesticated ruminants. Ruminants are further classified as grazers, browsers or intermediate grazers depending on their foraging behavior. Ruminants production systems are helpful in converting humanely inedible feedstuffs, pasture, and crop residues into high-quality edible food for humans. Additionally, ruminants production can thrive in poor and erodible lands where cultivation of human-food crops may perform dismally (Oltjen and Beckett, 1996). However, the continued inclusion of human edible food resource in the ruminants' diets has attracted concerns on sustainability of already scarce resource bearing in mind that there is projected demand increment in the foreseeable future. In addition, efficiency into which the ruminants convert humanly edible food into meat and milk is of concern and it depends on the quality of feed intake. However, it is worthy to note that the resultant protein from ruminant production is of higher quality and with a higher biological value than that in the substrate diet (Oltjen and Beckett, 1996).

The current ruminants production dynamism is expected to retain its vitality in the foreseeable future due to the increasing demand for food of animal origin. For instance, ruminant production contributed approximately 29 percent of global meat production in 2010 which was estimated to be about 81 million tons; out of the year total global production, about eighty percent was bovine meat. In the same year, the global milk production totaled to 717 million tons and cattle production contributed about 83 percent (Opio *et al*., 2013). The demand escalation is imminently expected and will be prompted by population growth, urbanization, and income increment. As a result, ruminant production systems are compelled to yield more in a contextual framework of increasing natural resources scarcity, food/feed crisis, and under stringent GHGs emission mitigation measures.

While ruminants play invaluable roles such as the provision of high-quality protein source to humans and source of income to livestock farmers, they also negatively affect the environment by being one of the important anthropogenic GHGs emitters. Emission of GHGs by ruminants comes from rumen fermentation, following fermentation, the gaseous products which include CH_4 are eructated by ruminants to the environment. With a significant ruminant production increment, enteric CH_4 emission is also expected to increase. Hence, to mitigate this dicey sequel of increased accumulation of GHGs, abatement strategies must be established and executed.

2.2 Rumen fermentation

Ruminants exhibit a symbiotic relationship with their gut microbiome which aid the host animal by producing a wide range of complex plant carbohydrates such as cellulose and hemicellulose-digesting enzymes yielding beneficial energy to both the host animal and symbionts microbes (Knapp *et al*., 2014). This implies that in ruminants, the ingested feed follow two distinct stage process which entails the following; the ingested bolus is enzymatically degraded producing various monomers such as sugars, amino acids and fatty acids and ultimately, the fermentation of the resultant monomers by symbiotic microbes which includes protozoa, fungi, bacteria and methanogenic archaea (Julian *et al*., 2016).

The ruminal fermentation occurs under highly controlled anaerobic ecosystem of 38-42^{0}C, pH 6-7, and redox potential -300 to -350mV (Julian *et al*., 2016). These conditions maintain an optimal functionality of ruminal microbes. Under the aforementioned anaerobic conditions, rumen fermentation of the ingested plant material occurs swiftly than in other anaerobic ecosystems and the products of fermentations are disparate. Rumen microbes have evolved to differ with other microbes of other anaerobic ecosystems. For instance, rumen protozoa do not exist in any other environment whether anaerobic. Although some genera of methanogens have been identified in other environments, but they remain to be totally different. Intriguingly, there is chasm between methanogens found in hindgut and forestomach in terms of population structure, metabolism and ecology according to Knapp *et al*., (2014).

Feed is physically broken into small pieces through mastication as the animal grazes or feed from the feed trough. Afterward, the swallowed boluses are regurgitated for further re-chewing into smaller pieces and further mixed with saliva containing digestive enzymes as part of preliminary processing. Once the boluses are re-swallowed, they are mixed with various liquids, mixed and maintained under the aforestated anaerobic conditions. The ingesta is acted upon by a massive community of rumen microorganisms ranging from bacterial, fungal, archaeal, and protozoal species to yield monomers and oligomers which are further fermented to produce VFA (acetate, propionate, and butyrate), ammonia, carbon dioxide, and hydrogen (Janssen, 2010). Rumen fermentation products such as VFA are absorbed across the rumen wall to provide the host animal with energy, amino acids, and ammonia which are the products of protein and non-protein nitrogen degradation and are in turn utilized by the rumen microbes to yield microbial protein. Collectively, the resultant microbial protein and ingested plant protein form dietary amino acids sources of the host animal. Tiny-unutilized feed particles suspended on the rumen liquor and microbes are discharged from reticulorumen to abomasum and lower animal's digestive tract by peristalsis for further enzymatic digestion and absorption. Essentially, the prime roles of rumen's microbiome are to help in pre-digestion of ingesta prior entering abomasum and conversion of feed components into VFA and microbial proteins (Janssen, 2010).

Julian *et al*., (2016) clearly described how gaseous products of fermentation which include; CO_2, ammonia (NH_3), and hydrogen (H_2) are released from rumen differently. Firstly, the gasses are released to rumen's headspace and eructated to the environment but some minute quantities are circulated to lungs for respiration. As for H_2, it is either cleared through VFA production or converted to methane. The latter process is the most probable route of H_2 clearance from the

rumen. Conversion of H_2 to CH_4 is facilitated by methanogenic Archaea because they obtain energy from the process. Total H_2 pool is small and invariably the concentration of dissolved H_2 ranges between 0.1 to 50 micrometer. The rate and amount of CH_4 formation are dependent on the rate and amount of H_2 that passes through the pool as described by Janssen, (2010).

The dissolved hydrogen gas in the rumen liquor depicts a fascinating dynamism depending on the feed type, time elapsed ensuing feeding, and passage rate of feed in the digestive tract. Report by Smolenski and Robinson, (1988) on forage-fed cattle showed that concentration of H_2 in the rumen increased steadily to reach to the peak concentration of up to 20 □M forthwith after feeding. The same trend of higher H_2 concentration ensuing feeding was reported on grain-fed ruminants by Robinson *et al*., (1981) where they found a 10-fold of H_2 concentration increment one hour after feeding which then steadily stabilized to normalcy. Passage rate of ingesta in the digestive tract is highly dependent on the degradability of the feed, if it's readily digested, then it swiftly passes through the digestive tract and vice versa is true. The passage rate of feed has been explicitly shown to affect H_2 concentration in rumen liquid as reported by Czerkawski and Breckenridge, (1971) in their study of sheep fed readily fermentable molasses sugar beet pulp.

H_2 concentration in the rumen is known to have direct influence on CH_4 formation. H_2 concentration in the rumen is invariably low during feeding but it starts to burgeon as feeding continues, during that time CH_4 as the by-product is usually low. It has been intimated that increased H_2 concentration in rumen results in a change of pathways to one that produces less H_2 and more propionate. Propionate formation is considered as an alternative to H_2 formation pathway since both pathways are characterized by electron acceptance (Baldwin *et al.,* 1963). Propionate formation entails a reduction of pyruvate to propionate while H_2 formation, H^+ protons are reduced to H_2. H_2 forming pathways' thermodynamics are regarded as somewhat inauspicious at high H_2 concentration than other pathways. In such a scenario, electrons from fermentation process are directed to pyruvate reduction to propionate and hence less CH_4 is yielded per united feed undergoing fermentation. On the other hand, if H_2 concentrations are low, more H_2 is formed, and in turn, less propionate is formed and eventually more CH_4 (Janssen, 2010).

However, it is worthy to note that rumen is not habitually uniform, some variations occur that tend to create transient microenvironments depending on the structural composition of the ingested food in those microenvironments and uneven mixing of ingesta among others. Hence, there will be regions with high or low H_2 concentration, thereby experiencing different pathways. Additionally, methanogens growth rate and load substantially vary along those microenvironments. Hence, cumulative effects of all those small and transient systems and changes across the rumen imply net resultant H_2 formed and therefore net ruminal CH_4 formed.

2.3 Ruminants contribution to environmental pollution and global warming

Livestock production plays an indispensable role in the general human livelihood but also have grievous environmental impacts. Ruminants production has significantly contributed to land degradation, water pollution, loss of biodiversity, and climatic change. Traditionally, ruminants production was somewhat small and incorporated in subsistence kind of farming and positively contributed to the conversion of by-products and other inedible materials into edible products and rendering other good and services. However, that kind of production has markedly changed to a demand-driven production as opposed to the previous supply-driven production (Gerber *et al*., 2013). With the trend set to continue, ruminants production's environmental impacts have increasingly become the cynosure of the scientific community who are trying to evaluate how high ruminants production can be sustained under stringent environmental impacts mitigation measures.

Climatic change in the last one decade has been outrageous and has been regarded as the warmest decade in the history, with the year 2005 and 2010 being ranked as the hottest. In fact, back in 2012, World Bank, (2012) warned that the world was on the track of a 4^0C warmer world and warned about the disastrous sequels that change would have. It urged all the stakeholders that dire responses have to be opted to stabilize the scenario citing that the later the reduction strategies start, the much effort is required or the set target of 2^0C by 2027 become elusive.

It is explicitly recognized that the increased concentration of GHGs in the atmosphere is responsible for global warming. The main GHGs include water vapor, CH_4, CO_2, nitrous oxide (N_2O), and fluorinated gasses such as chlorofluorocarbons. Greenhouse effect occurs since GHGs allow shortwave to pass through the atmosphere to the earth surface which then reradiates longwave radiation that is ensnared by GHGs in the atmosphere. The captured long waves heat the atmosphere which in turn reradiates the long waves in all direction, earth surface included. GHGs are expressed often as CO_2–equivalents (CO_2-eq); CH_4 being the third most abundant trace gas in the atmosphere trailing behind water vapor and CO_2. Additionally, CH_4 half-life in the atmosphere is about 12.4 years and its global warming potential of about 23 times (Thorpe, 2009).

Livestock production activities have been alleged to contribute about 18 percent of the total anthropogenic GHGs quantified in CO_2-eq thus its contribution totaling to over 80 percent of agricultural GHGs emissions globally (FAO, 2006). CH_4 from enteric emission constituted about 32 percents of non-CO_2 emissions from agriculture in the year 2005 (Smith et al., 2007). Enteric CH_4 emission has been increasing over the last couple of decades and the concentration of CH_4 in the atmosphere is expected to exacerbate if enteric CH_4 emission continues to increase in proportion to the projected increment of ruminant numbers then CH_4 is expected to increase by an astounding percentage of even up to 60 percent in general livestock production (FAO, 2003).

In addition to global warming concern, enteric methanogenesis represents one of the feed conversion inefficiencies as it results in energy loss from 2-12 percent of the gross energy intake (Johnson and Johnson, 1995). However, it is worth noting that in addition to enteric CH_4 emission, ruminants manure contributes substantially to CH_4 emission in the environment. Out of the total livestock CH_4 emitted, statistics show that 83 percent is contributed by enteric

fermentation and astoundingly, ruminants are predominant contributors by about 98.7 percent. On the other hand, CH_4 emanating from manure management just accounts for about 17 percent of the total CH_4 emitted from livestock production in which cattle contribute about 44.7 percent of the total CH_4 emanating from manure (FAO, 2006). Enteric CH_4 emission can be influenced by various factors as delineated by Shibata and Terada, (2010).

2.4 Factors that influence enteric methane emission

Thre are a number of factors that influence CH_4 production in ruminants, these factors form the basis of the avenue through which abatement strategies can be effected, they include the following;

2.4.1 Feed type and composition

The quality of the feed ingested by ruminants largely influences CH_4 production and emission by ruminants. In the rumen as described previously, degradation of ingesta is highly dependent on microbial activities. Feed composition will significantly affect rumen microbes' activities and eventually CH_4 production. CH_4 production has depicted a propensity to increase if the diet fiber content is increased and on the other hand, decrease as feed's protein content increases (Johnson and Johnson, 1995). Feed processing which involves chopping has been shown to affect CH_4 production; the finer the choppings the lower CH_4 production is. This scenario is usually ascribed to the increment of digested cellulose that normally contributes to more CH_4 production than other carbohydrate components. Generally, CH_4 production tends to be lower in high-concentrate feed and higher in the high-roughage feed (Lovett *et al*., 2003). Any auspicious condition that leads to rumen microbial growth and increased propionate production will eventually result in reduced CH_4 production.

2.4.2 Feed intake

Enteric CH_4 emission is highly dependent on the feed intake and digestibility. CH_4 being a product of rumen fermentation, if the fermentation is rapid, then low CH_4 production will occur as reported by Johnson and Johnson, (1995). However, they noted that if the proportion of highly digestible carbohydrates in the diet is low, then higher CH_4 production will occur. CH_4 emission and feed intake have been reported in several studies to have an inverse relationship; increase in feed intake decreases CH_4 production per unit feed ingested (Hart *et al.,* 2009). Scholars have tried to corroborate this notion by ascribing it to the brief ingesta retention in the rumen and hence results in less extensive fermentation. This scenario favors more propionate production and less H_2 production thereby reducing CH_4 produced per unit feed ingested as previously described by Janssen, (2010).

2.4.3 Forage preparation and preservation method

The method of forage processing and how it was preserved has been shown to affect CH_4 emission. There is a tendency of a lower enteric methanogenesis if the forage was ensiled as opposed to the dried one. Further, pelleting or chopping has also exhibited an influence on the level of CH_4 production and emission; finely chopped feed leads to a lower CH_4 emission than coarsely chopped (Martin *et al*., 2010). This has been attributed to the decreased ruminal availability of the organic matter and swift passage rate of digesta in the rumen. Although

feeding of finely grounded forage at high intake has been reported to significantly influence the reduction of CH_4 emission by even up to 40 percent hence appearing to be a plausible way of CH_4 emission mitigation, it would significantly reduce farmers' profit margin.

2.4.4 Feeding level

The level of feeding has been defined as the amount of feed consumed divided by animal's maintenance feed requirements. The feeding level usually affects digestibility of feed; as feeding level increases, digestibility reduces. A Higher level of feeding leads to decreased enteric CH_4 emission, this has been attributed to the fact that less energy in feed at a higher feeding is available for digestion. Allard, (2009) corroborated this notion in an experiment where the proportion of energy lost and CH_4 yield decreased as the level of feeding increased.

2.4.5 Systems of feeding

A study by Yurtseven and Ozturk, (2009) which evaluated two systems of feeding, choice feeding and conventional system on CH_4 emission found that choice feeding results in reduced CH_4 emission. They attributed that to swift passage rate of digesta and the rate of digestion. They intimated that the system favors propionate production over acetate thus limiting H_2 production. Additionally, Total Mixed Ration has been showed to decrease CH_4 production as opposed to separate forage-concentrate feeding system (Sejian *et al.,* 2011).

2.4.6 By-products inclusion in the diet.

Due to the possession of rumen microbiome, ruminants have the ability to utilize fibrous by-products and convert them into humanly edible products. The volume of ruminal gasses emitted widely vary from one by-product to another depending on the cell wall and cell content. This implies that by-products with high structural carbohydrates content ferment slowly as opposed to those with considerably more non-structural carbohydrates thus yielding more methane per unit feed fermented. This has been attributed to higher acetate: propionate ratio (Maheri-Sis *et al.,* 2007).

2.4.7 Forage species and maturity

It is widely known that forage maturity affects CH_4 production, with CH_4 production increasing as forage matures. Further, Legume-grasses are known to yield less enteric CH_4 during fermentation than grass forage (McAllister *et al.,* 1996). This has been ascribed to less structural carbohydrates content in legume than in grass forage hence rapid fermentation which leads to more propionate production as previously indicated by Johnson and Johnson, (1995).

2.4.8 Proportion of concentrates in the diet

It is widely known that the proportion of concentrate in the diet has a great influence on the amount of CH_4 produced during enteric fermentation. The increment of dietary concentrates proportion normally reduces enteric CH_4 emission. Yan *et al.,* (2000) reported that increased concentrates proportion lead to a reduction of CH_4 emitted due to shifting of increased propionate production and reduced acetate production, rumen pH also reduces and the resultant condition poses an inauspicious environment for methanogens which are sensitive to low pH. However, increasing the proportion of concentrates in the diet has a limit to preempt clinical

acidosis and to strike a balance between energy intake and animal requirements to avoid overfeeding which can be uneconomical especially in low producing ruminants.

2.4.9 Feed digestibility

Generally, the feed quality is assessed in terms of its digestibility, with a good quality feed having a higher digestibility. Feed digestibility have been shown to influence enteric CH_4 emission, where the increased feed digestibility has a cumulative CH_4 emission reduction effect in animal's lifetime as reported by Hart *et al.,* (2009). They noted that as the feed digestibility increases, energy availability to the animal increases which triggers a higher animal growth rate. As a result, the amount of CH_4 emitted per unit of production decreases as feed digestibility increases.

Understanding of the above factors that has a great influence on enteric CH_4 emission forms a basis of envisaging apt, economical, sustainable, and healthy mitigation measures. Some of the above measures, even though they seem to possess pronounced potential towards the reduction of enteric CH_4 emitted, they equally possess limitations. For instance, if we leverage some, there is the possibility of exposing ruminants to clinical conditions such as acidosis in the case of increasing dietary concentrates proportion in the diet. Some would have increased cost implications especially if the stock is not a high producing one, while others may not be practically sustainable.

However, scientists and all stakeholders have for long been engaging dogged determination to come up with various beneficial and sustainable enteric CH_4 abatement methods. The most important consideration when investigating apt mitigation methods is that they are supposed to be cost-effective in order to gain acceptability from the farmers. Generally, all the proposed methods revolve around three broad categories such as feeds and feeding management, use of rumen modifiers, and increasing animal production.

This dissertation will delineate such methods that have possibly been thought to abate enteric methane emission. However, all methods may not have been tested in one ruminant species but it is generally regarded that applicability of such strategies is somewhat the same irrespective of the animal in which they were tested in. The methods include the following;

2.5 Enteric methane emission mitigation measures

2.5.1 Nutritional aspects

Nutritional mitigation measures revolve mainly around the choice of ingredients that will modify VFAs production patterns. Feeding better quality feeds that will improve the animal performance and targeting to increase passage rate of digesta in the rumen.

2.5.2 Feed and feed management

Substrates for rumen microbial fermentation are provided by feed ingredients. The quality of such ingredients modifies the energy obtained by rumen microbiome and the pattern of VFA formation and eventually, CH_4 produced. The proportion of VFA formed during microbial fermentation is crucial in determining the amount of CH_4 produced. Hence, any inclusion of

dietary component that will cause VFA formation pattern shift in favor of propionate formation will eventually lead to lower enteric CH_4 emission (Van Nevel and Demeyer, 1996).

Protein degradation in the rumen and subsequent microbial protein formation either results in consumption or production of H_2. Fatty acids biodehydration can be used to provide an avenue for H_2 consumption in the rumen. Additionally, any mechanism that can favor variation of nitrogen metabolism in rumen will consequently result in a significant reduction of CH_4 production.

Okine *et al.,* (1989) reported that CH_4 production is inversely correlated with the passage rate of ruminal particulate matter. Hence, an expedition of substrate passage rate in the rumen will limit the extent of microbial degradation, affect the rate of microbial growth and also alter VFA formation pattern. The aforementioned effects result in the reduction of CH_4 production and emission.

Dry Matter Intake (DMI) have exhibited an intriguing effect on CH_4 production in that total CH_4 production increases as DMI increases but production curve interestingly shifts above maintenance level so that it starts decreasing with increasing DMI (Moe and Tyrrell, 1979). Studies have confirmed the aforementioned correlation between DMI and CH_4 production when ruminants are fed *ad libitum*. Additionally, the increasing DMI and milk production have been found to have CH_4 reduction impact. Hence, any milk production increment would somehow trigger lower CH_4 production.

The digestibility of feed ingredients has a huge impact on CH_4 formation. For instance, CH_4 production is significantly affected ensuing depression of ruminal starch digestibility with intake increment. Firkins *et al.,* (2001) reported that ruminal starch digestibility decreased as DMI increased. That meant that any starch that eluded ruminal fermentation was digested in small intestine other than being fermented in the hindgut. That evasion was favorable to an animal in terms of energy than when fermentation had occurred and VFAs are formed. Although there was no net energy difference whether starch was fermented in the rumen or digested in the small intestine as reported by Huntington *et al.,* (2006), but there was net CH_4 production reduction. Hence, any measure that will result in the increment of the passage rate with an increasing DMI and reduce the extent of substrate degradation in the rumen will significantly reduce CH_4 production and the energy at disposal for milk production will eventually increase. To achieve the aforestated, several attributes of the ration must be meticulously adjusted and altered. Such aspects include the following;

Type and source of carbohydrate must be put into consideration if CH_4 production reduction is the prime intention. Feeding ruminants with more or readily digestible carbohydrates leads to a greater DMI, passage rate, and lesser CH_4 formation. This is because rumen fermentation end-products especially VFA are greatly influenced by proportionality of Neutral Detergent Fiber (NDF) and Non-Fibrous carbohydrate (NFC) in the diet fed (Moe and Tyrrell, 1979). Forage type and region of propagation have been reported to have an influence on CH_4 production by Archimede *et al.,* (2011). They reported that due to higher NDF content and excessive lignification, tropical grasses are less digestible than temperate ones and hence their fermentation

yield more CH_4. They also further noted that although tropical legumes are less digestible, they lead to less CH_4 production. They attributed that finding to high tannins and secondary metabolites in tropical regumes that affect NDF digestibility and eventually CH_4 production. From the study above, it is evident that use of warm climate legumes and temperate grasses may help in reducing CH_4 production.

Feed quality, species, harvesting time and storage of forage must be highly considered if the reduction of CH_4 produced is to be achieved. About 75 percent of global ruminant enteric CH_4 emission emanates from ruminants feeding or grazing on poor quality feed (Leng, 1993). So to effectively abate enteric CH_4 production, ruminants must be fed good-quality feeds. This is achieved by feeding them with less-mature forage, proper selection of forages that have good digestibility and proper storage such ensiling to preserve digestible nutrients. Accrued benefits will exceed environmental benefits since feeding good-quality forage is also profitable to farmers.

Feed preparation prior feeding which includes chopping, pelleting, and grinding has been highly associated with enteric CH_4 reduction depending on the feed involved. Such processings alter NDF digestibility in the rumen, heighten the passage rate and increase propionate: acetate (P:A) ratio (Van Nevel and Demeyer, 1996). However, Johnson and Johnson, (1995) pointed out that for processing effects to be pronounced, DMI must not be restricted as the effect is evident with increasing intake levels. Pelleting was quoted having a high possibility of reducing CH_4 emission greatly than chopping. However, Hironaka *et al.,* (1996) accentuated that the extent of CH_4 reduction by pelleting does not corroborate the necessity of energy expenditure in pelleting. Additionally, pelleting is only beneficial and produce a marked CH_4 reduction in poor-quality forage and precise care must be taken to preempt ruminal acidosis or fine pelleted particle eluding rumen fermentation. To compensate for the feed processing shortcomings, treatment of poor-quality straws with alkali has been shown to contribute a significant CH_4 reduction as reported by Van Nevel and Demeyer, (1996).

Lipid and fatty acids supplementation have been largely associated with enteric CH_4 reduction. However, the effects of dietary lipid inclusion in the ruminant diet on CH_4 production are highly dependent on the composition of the diet, the source of the lipid, inclusion percentage of DMI, and fatty acids profile as indicated by Beauchemin *et al.,* (2007). Lipids inclusion in the ruminants diet have shown to trigger a substantial CH_4 reduction, the reported reduction was associated with dilution of fermentable carbohydrates in DMI and reduced total tract NDF digestibility and this had an eventual reduction of DMI (Hollmann *et al,* 2013). They pointed out that inclusion of dietary lipid especially coconut oil reduces CH_4/ECM (Energy-Corrected Milk) but at an expense of reduced DMI which could prove to be a bit dicey over the period of time in terms of energy balance and milk production. Unsaturated fatty acids inclusion in the ruminants diet have been showed to act as a sink for H_2 produced during fermentation which acts as a precursor for CH_4 production. During biohydrogenation, H_2 is ensnared by double bonds of unsaturated fatty acids hence limiting H_2 available for CH_4 production.

2.5.3 Genetic approaches

Genetic improvement of ruminants has lately been demonstrated to have the potential to increase productivity while reducing CH_4 production especially CH_4/ECM. Several ruminant attributes that contribute to CH_4 emission such digesta retention in rumen, host-microbiome interface and plant species/maturity selection while grazing are highly considered to be heritable and if the alteration is done, less CH_4 can be emitted per DMI and productivity increased.

There exists a general consensus among many scholars that increment of productivity will significantly contribute to reduction of CH_4 emission per unit of products. Moss *et al.,* (2000) clearly demonstrated how the improved ruminants' productivity will result in a significant reduction of CH_4 emitted. Achievement of ruminants production efficiency can only be through genetic selection coupled with proper animal husbandry practices and effective nutritional attributes like the one described above. Genetic selection in the dairy industry has made perceptible strides in the last couple of decades, for instance, genetic selection in Northern America has resulted into over 400 percentage increment in the last six decades with a substantial CH_4 emission decline (Capper *et al.,* 2009).

To achieve CH_4/ECM reduction in an individual dairy cow, some genetic selection measures must be undertaken. Some of the measures include; an increment of milk production per cow with the slightest DMI increase which ensures Gross Energy (GE) efficiency and selection of Residual Feed Intake (RFI). These selection tactics are based on the notion that reduction of the proportion of GE used for maintenance while either maintaining or increasing milk production would have an eventual CH_4/ECM reduction. The pronounced genetic selection has been inclined towards improving productivity, but less has been done on RFI. This entails genetic selection of cows that efficiently use feed ingredients for milk synthesis at fixed body size. Although RFI serves as a new way of understanding the physiological mechanism behind variation in feed efficiency, its heritabilities have been rated too low in a dairy cow.

Additionally, genetic selection contributes to CH_4 reduction indirectly by prolonging the lifetime of the herd as well as increasing its productivity through improvement of disease resistance, heat-stress tolerance, and improved reproductive performances. Although genetic approaches are somewhat promising in capitalizing on heritable traits in dairy cows to reduce enteric CH_4, they are not fully developed to demonstrate the direct effect on CH_4 reduction plus they must be adequately supported by nutritional management to actualize CH_4/ECM reduction.

2.5.4 Use of rumen modifiers and feed additives

Up to date, myriads of rumen modifiers and feed additives have been studied and revealed potential to reduce CH_4 emission. However, the mode of action of each disparately varies from each other. The mode of actions of such modifiers and feed additives range from offering a competitive pathway for rumen produced H_2, protozoa suppression and/or obstructing

methanogens or decimating methanogens. An extensive research has been carried out on a number of chemical additives which have shown potential to reduce CH_4 emission especially in an *in vitro* setup. The usage of chemical feed additives and antibiotics has been greeted with mixed reactions from stakeholders. Some, when included into the experimental diets in *in vivo* tests either yield totally different results or the reported CH_4 reduction effect is transient. The usage of others such as antibiotics has been banned in some region such as the European Union in 2006 while the general public has lately developed a predilection for natural additives over chemical additives. All the aforementioned shortcomings of chemical additives in CH_4 reduction and unacceptability by stakeholders have spurred scientist to delve into alternative strategies to fulfill their pursuit to abate enteric CH_4 emission. These natural additives include the following as described by Wallace *et al.,* (2002) plant containing bioactive compounds such as saponins, tannins, and essential oils. These compounds are generally regarded as plant secondary metabolites or phytochemicals since they are non-nutritive metabolites but vital for survivability of the plant. Although their mode of action has not clearly dawned on the scientist, some are reported to being toxic to methanogens such as tannins or ciliated protozoa such as saponins while essential oils have various effects on methanogens. Bodas *et al.,* (2012) described individual impacts of metabolites on CH_4 reduction as the following;

2.5.4.1 Tannins

Extensive researches both *in vitro* and *in vivo* have revealed that tannins from a wide range of sources have a vast potential to reduce CH_4 formation, be it condensed or hydrolysable. The efficacies of tannins on CH_4 reduction has been largely dependent on tannins source and molecular weights in case of condensed tannins. Although as earlier stated, the mode of action is not clearly apprehended but they are highly believed to be antimicrobial metabolites which act through bacteriostatic activities to inhibit various ruminal microbes such as methanogens, protozoa, and cellulolytic bacteria as reported by Tavendale *et al.,* (2005). Hydrolysable tannins depict somewhat direct effect by inhibiting methanogens while condensed tannins exhibit an indirect effect by reducing fiber digestion. Goel *et al.,* (2011) reported this juxtaposition of the two type of tannins.

2.5.4.2 Saponins

Saponins or plant rich in saponins compounds have been shown to have a significant effect on CH_4 reduction by various studies. 10-25 percent enteric CH_4 reduction ability of saponins have been reported. The reduction is because of saponins' potent anti-protozoa that lead to the formation of complexes with protozoa cell membranes' sterols as was reported by Goel *et al.,* (2011) thus reducing CH_4 production. Studies have reported that at even low concentration, saponins are effective as antiprotozoal compounds. Additionally, saponins have been shown to only affect methanogens at high concentration (Wina *et al.,* 2005). On the other hand, saponins have been reported to limit H_2 availability in the rumen for methanogenesis.

2.5.4.3 Essential oils

These are regarded as aromatic lipophilic compounds where all contain chemical constituent and functional groups such as phenols and terpenoid. Due to their lipophilic attribute, they have a high affinity for microbes' cell membranes. Essential oils' functional groups usually interface with the cell membrane of rumen microbiome. These complexes formation between essential oils and rumen microbiome obstruct electron movement across the microbial cell membrane, which greatly affects rumen microbes' activity. This obstruction of methanogens' activity and change to rumen archaea are thought to contribute to the reduction of CH_4 (Benchaar and Greathead, 2011).The effect is more pronounced in gram-positive than in gram-negative bacteria. Though essential oils seem to be promising in enteric CH_4 reduction, some concerns have been raised about sustainability and usage of essential oils to reduce CH_4 emission. Reduction of CH_4 production normally occurs at a higher level of inclusion which may affect rumen fermentation and feed digestion especially in *in vivo*. Additionally, rumen microbes tend to adapt swiftly to essential oils exposure hence casting doubts on essential oils efficacy as highlighted by Benchaar and Greathead, (2011).

2.5.4.4 Microalgae

As aforementioned, utilization of microalgae such as *Euglena gracilis* as a biofuel source and as a supplement to both humans' foods and animals' feeds has gained traction in scientific research. This spurred us to delve into the utilization of Euglena as a supplement to animals' diet and evaluate its effect on nutrients intake, digestibility, nutrients balance, and CH_4 emission.

Briefly, Euglena is a genus of single-celled, free living, fresh-water flagellated organism that depicts both plants and animals' characteristics. Euglena has an intriguing growth systems such as photoautotrophically, photoheterotrophically and heterotrophically as described by Fujita *et al.,* (2008). That is, it is able to use photosynthesis and heterotrophic oxidative assimilation as interchangeable sources of carbon and energy, hence regarded as mixotroph. Most species of Euglena are generally known as obligatory photoautotrophs since they hardly grow in inorganic media and require a certain source of nitrogen and amino acids to grow effectively. Most euglenoids' growth is expedited by either addition of lower organic acids or lower alcohols in some species. This was in concordance with Tani and Tsumura, (1989) findings on the impact of the addition of nitrogenous compounds and supplementation of Euglena growth with alcohols.

Anatomically, euglenoids' body is a fusiform, that is, anteriorly rounded and posteriorly tapering gradually to form a tip. Lengthwise, they vary from 40 to 60 micrometers while diameter ranges between 10-18 micrometer. Euglena possesses a flagellum whose length commensurates with body length or longer. They also possess chloroplasts which vary in numbers but are star-shaped. In addition, they have paramylon which are rod-shaped bodies that store carbohydrates synthesized by chlorophylls and mostly surround chloroplasts. Movement of euglenoids is by contraction which is also known as metaboly, where the unicellular organism contorts to a spherical-like body then ensued by swift expansion through peristaltic-sort of motion. The effectiveness of this movement is highly dependent on pellicle's elasticity.

The world is increasingly facing myriads of hurdles to solve in the current and coming decade, they include; increasing CO_2 emissions, burgeoning demand for quality food/feed, increasing

demand for biofuels which has imposed strain on corn production. This has spurred the stakeholders to seek alternative measures to ward off the exacerbation of the current and already alarming situation. Euglenoids are the organisms touted to have a combination of all those attributes that can aid in mitigating all those dicey hurdles.

Nutritionally, *Euglena gracilis* has vast nutrients; It is reported to have over 59 nutrients by Euglena co.ltd, a Tokyo-based company. Researchers have concurred with such findings, for instance, Tayekama *et al.,* (1997) and Barsanti *et al.,* (2000) have reported various nutrients that the algae have. They indicated that it has a high content of protein, polyunsaturated fatty acids, various antioxidants such as vitamin C, E, □-carotene. These antioxidants are vital in preventing oxidative stress damaging biological membranes. The unicellular flagellates are known to be a good source of □-1,3-glucan paramylon and alpha-tocopherol, which is regarded as the most biologically active vitamin E form. Worldwide demand for alpha-tocopherol is increasing and is reported to be almost overcoming the limited supply of synthetic alpha-tocopherol supply. However, Tani and Tsumura, (1989) suggested that *Euglena gracilis* can replace former sources of alpha-tocopherol due to the high content of RRR-□-tocopherol.

Energy-wise, the current petroleum reserves are dwindling and combustion of such fossil fuels emits a humongous amount of CO_2. Previously perceived sources of biofuels are currently strained by food/feed crisis and increased demand for both resources. Hence an alternative has to be sought, the source must be environmentally benign and sustainable for both consumers and industrial usage. To mitigate that concern, there are alternative sources of energy such as solar, wind, geothermal and biomass. However, very few exhibit both sustainability and economic feasibility, the best being biofuel from available biomass. Biofuels being the gaseous or liquid fuels from biomass which is vegetable matter obtained ensuing photosynthesis. The prime characteristics being biodegradability, sustainability, renewability, carbon neutrality and hence environmentally benign. Algae's biofuels production is hugely considered as most efficient, environmentally friendly and sustainable. John *et al.,* (2011) confirmed that algal biomass and industrial waste can be efficiently utilized to produce environmentally benign fuel bioethanol. Additionally, algae contain oil and also vast carbohydrates content that can be used as bioethanol's raw material.

The algal biofuel production offers unparalleled merits over the other sources and over fossil fuels. Firstly, they do not conflict with food production. Secondly, algae as biofuel raw material produce over 300 times lipid than traditionally used crops for biodiesel production, this production level can further be increased by changing the medium composition. Thirdly, Algal production depicts a rapid reproduction rate, doubling over 24 hours. This ensures sustainability as they can be harvested over once in a year. Lastly, they aid in carbon sequestration and also algae biofuel does not contain sulfur, it is non-toxic and highly biodegradable. These depict its environmental-friendly characteristics. All the aforestated advantages of algal biofuels have made microalgae being the most promising source of biofuels as described by Schenk *et al,* (2008).

Euglena gracilis as microalgae have huge potential as food and feed supplements. If the stakeholders leverage the potential, it can help in mitigation of the anticipated increment of

demand for quality food/feed. Klein and Buchholz, (2013) accentuated the potential possessed by microalgae to meet the expected demand increment for food/feed and development of nutraceuticals. They emphasized that due to the high concentration of polyunsaturated fatty acids, then microalgae are valuable nutraceuticals. Studies have confirmed that euglenoid production is a new way of producing proteins and value added metabolites of biotechnological benefits (Kraj□ovi□ *et al.,*2015). They indicated that storages such as polysaccharides, paramylon has immunostimulatory attributes, □-1,3-glucan has anti-tumor properties and Euglena biomass can be used for aquaculture and animal feed's supplements due to their nutrients content. Their findings are in agreement with Euglena co.ltd, a Tokyo-based company which has extensively researched about the possibilities of Euglena being a food/feed supplement. Euglena possesses vast nutrients and high protein content which present potential of being used as a supplement to both feed and foods. For instance, an extensive research conducted by the company revealed that the inclusion of Euglena in fish and poultry feeds had a positive impact as it bolstered survivability of fingerlings and increased taurine content in chicken meat. Further, the company has suggested that the utilization of defatted Euglena as a feed supplement would ensure full utilization of Euglena. This is because of high protein content that the residue has and also the exceptional high digestion-absorption rate associated with Euglena. Euglena exhibits high digestibility due to lack of cell wall which necessitates availability of cellulase to degrade it.

All the above Euglena exceptional nutritive properties spurred this study to conduct an extensive research on Euglena as a ruminants' feed supplement. The study had the intention of evaluating various effects of Euglena inclusion in the ruminants diet on nutrients intake, digestibility, nutrients balance, rumen ecosystem and CH_4 emission.

3 MATERIALS AND METHODS

3.1 Study area:

The experiments were carried out in the department of animal and environmental hygiene's laboratories and field science center of Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan which lies in 42.8760° N, 143.1732° E coordinates. The experiments, the use four fistulated Corriedale sheep and two fistulated non-lactating Holstein-Friesian cows were approved by the university's committee for animal welfare and ethics.

3.2 The nature of experiments

The experiments were categorized into two major categories; *in vivo* and *in vitro.*

In vivo: The experiment was conducted to evaluate all the effects of Euglena inclusion in the animal diet except its effect on CH_4 emission, which was limited by the fact that the rams had fistula which is said to leak some fermentation gasses by other studies and in addition, the present study intended to evaluate fermentation parameters and hence it could not use non-fistulated rams.

In vitro: The experiment was conducted to evaluate all parameters including CH_4 emission which was conducted by use of continuous fermentation and continual gas quantification system.

3.3 In vivo

3.3.1 Animal diet

Powdered samples of pure *Euglena gracilis* were sourced from Euglena Co. Ltd., a Tokyo-based company. Following reception of *E. gracilis* samples, they were subjected to various chemical analyses before inclusion in the animal diet. Then, a pilot study was conducted to evaluate whether sheep would lick Euglena powder and general behavior during and after licking.

Klein grass (*Panicum coloratum*) hay was supplied by a local animal feed company and finely chopped to enhance intake. Klein grass is a warm-season, bunchgrass and perennial grass native to Africa. Its prime propagation period starts early in spring and grows until late in fall. Klein grass grows up to four feet tall, from the base up to about 20-45 cm being the erect fibrous stem.

Concentrate and mineral blocks were also sourced from a local animal feed company. It was subjected to various chemical analyses while mineral block had the following main mineral

composition per kilogram; yellow iron oxide (1742mg), iron sesquioxide (196mg), copper sulfate (377mg), cobalt sulfate (66mg) zinc sulfate (1235mg), manganese carbonate (1046mg), calcium iodate (77mg), sodium selenite (33mg) and common salt (971 g).

3.3.2 Sheep and feeding

Four previously fistulated Corriedale rams which weighed 38.81±4.38 kg were used for Euglena feeding trial. The rams were weighed at the inception of the experiment and at beginning of every new period. They were then individually moved into transparently walled feeding trial cages, arrayed next to each other with the distance between the cages being about one meter. The chambers had in them firmly fitted waterers and feeders, which each ram had an easy access to. The floor, which was a bit raised, had strongly welded mesh wire that could allow an uninterrupted passage of both droppings and urine which were collected by an inclining underneath fitted plastic container. The approximate housing of the experimental rams in such clear chambers is said to hasten the acclimatization process and also significantly lessen the tendency of ram being distressed which could affect DMI. Additionally, the rams have previously been used in such a setup severally while conducting somewhat similar studies. This also aided in prompt acclimatization and obviated any form of even mild distress among the rams. The rams were fed at maintenance level with a basal diet of Klein grass twice daily early in the morning (9:00 am) and in the evening (4:00 pm). Concentrate and Euglena were always thoroughly mixed to enhance intake and obviate Euglena refusal. Initially, at the inception of the study, sheep were randomly allocated to either of the four treatments. The treatments included the following; Treatment one (T1) which was control, that is, there was no Euglena supplementation to the basal diet fed to the sheep. Treatment two (T2), Treatment three (T3) and Treatment four (T4) had the following levels of Euglena inclusion per Dry Matter (DM) ration; 5, 10 and 15 percent respectively. However, to clearly evaluate the effects of Euglena inclusion, the diet was modified slightly to achieve isonitrogenous and isocaloric diet so as to ensure the resultant effects were not due to an increase of protein or energy but their source and levels in the diet. After the first period, the rams were subjected to the next level of inclusion systematically. That is, the sheep from T1 to T2, T2 to T3, T3 to T4 and T4 to T1. That order was followed until the end of the experiment. Water was provided in the waterers all through and the mineral block was provided throughout the experiment.

3.3.3 Experimental design and procedures

The experiment adopted a 4×4 Latin square design in order to evaluate the effect of Euglena supplementation in two directions. The experiment was conducted for 80 days during summer of 2015; its inception was in first July and completion in late September. The experimental period was equally portioned into four periods which consisted 20 days. Each period had the following routine; fourteen days of diet acclimatization, five days of feces and urine collection, and one

final day of rumen liquor collection. As mentioned earlier, the rams were weighed on the first day of every period to monitor whether there were weight variations. After 14 acclimatization days elapsed, feces and urine collection for five days took place and during the same period refusals were also collected.

In the evening of every fourteenth day of each period, the rams were fitted with clean and dry feces collection aprons and urine was collected in the clean five-liter buckets which had muslin cloth covering the brim to sieve and preempt entry of any debris into the bucket. Every day after emptying, cleaning and drying of the bucket, 100 ml of sulfuric acid was added into each bucket. The addition of sulfuric acid was intended to lower the pH of urine to below 3 in order prevent Nitrogen degradation by bacteria. Each day sheep's total urine collection was pooled in a bigger clean bucket, thoroughly mixed, weighed and recorded. Then, a 50ml representative urine sample was drawn from the pooled sample and transferred into a storage plastic bottles. The collected sample was then forthwith stored in a deep freezer at -20^0 C awaiting laboratory analyses. The procedure was repeated for all the other treatments' urine. The remnant urine was then aptly discarded and buckets thoroughly cleaned and dried for the subsequent day usage.

The whole or half-day feces were collected into the apron affixed to the body of each sheep and emptied into clear sealable collection bags, weighed and recorded. Forthwith, the feces were stored in a deep freezer maintained at -20^0 C awaiting completion of the collection period. Upon completion of collecting period, the frozen feces of all the five days for each treatment were thawed by exposure to room temperature for a while, then pooled into large clean crates, followed by even mixing. Afterward, a 200 g representative fecal sample was drawn from pooled mixed feces and oven-dried for forty-eight hours at 60^0C. After 48 hours of oven-drying elapsed, the dried feces were weighed again and finely ground to pass 1 mm sieve and packed in sealable aluminum foil bags for chemical analyses. The aforementioned set of procedures were followed for other treatments' fecal collection. The remaining unwanted feces were disposed aptly in the farm designated compost storage facility.

Throughout the experiment, there was only Klein grass hay refusal, concentrate-Euglena mixture was consumed completely. Every morning before feed provision, refusals were weighed and emptied into sealable bags. After the end of each period, the refusals of each treatment were as well pooled into a big crate, mixed evenly and then a representative sample was obtained and oven-dried for forty-eight hours at 60^0C. Then finely ground to pass through 1 mm sieve and stored in aluminum foil bags for the subsequent chemical analyses.

3.3.4 Rumen fluid siphoning

The rumen liquor collection was done during the last day of each period. Siphoning of rumen fluid began early in the morning, which was immediately after feed provision, and regarded as zero-hour collection. Rumen fluid collection continued after every two hours until eight hours elapsed, then the final collection was done at 24 hours, early the following morning prior the morning feeding. Rumen fluid collection was effected by use of rumen fluid sampler attached to a syringe. A different sampler would be used for each ram to prevent cross-contamination. The procedure followed involved the following; the fistula would be carefully opened and the sampler tube scrupulously and aseptically inserted into the middle of the rumen. After

ascertaining proper placement of the tube in the rumen, the dosing syringe would then be connected to the sampler tube. Then aspiration was done steadily and diligently to the full capacity of the syringe which was 20 ml. Then, the sampler tube would gradually and carefully be withdrawn from the ram's rumen. Finally, the fistula would then be tightly and aseptically closed. Forthwith, the aspirated rumen fluid would then be emptied into a collection vessel, pH measured, recorded and stored in a deep freezer at -20^0 C. At the 24^{th} hour of rumen fluid sampling, 1ml of rumen fluid from each ram, which represented treatments, was mixed with 4 ml of methylgreen-formalin-saline in 10 ml tubes, wrapped in aluminum foil and stored in dark chamber awaiting ciliates counting (Ogimoto and Imai, 1981).

3.4 In vitro

3.4.1 Study's substrate

This part of the study utilized Kikuyu grass (*Pennisetum clandestinum*), which was obtained from kipipiri district, central Kenya which lies in the following coordinates 0.4383° S, 36.5472° E . The area receives average annual rainfall of between 700mm and 1500mm, with two notable rainy seasons which include long rainy season (April-August) and short rainy season (September- November). Temperatures usually range between 12^0 C and 25^0 C. The area has a loamy soil cover. Kikuyu grass has been regarded as an aggressive and vigorous perennial grass due to its growth patterns. It spreads both underground and on the surface by stolons and long, narrow and hairy stem respectively. The grass normally exhibits compact leaves arrangements and flowering usually on short side shoots. The grass propagation is either by seeds or stolons and mature stems; where the propagation materials are scattered on a well-prepared land with fertile soil containing sufficient moisture.

The grass was harvested at the flowering stage from various locations of the constituency, transported to one collection point, cool-dried in a transparent walled structure for some days until satisfactory wilting was achieved. Then, the wilted grass was finely chopped, pooled in several sacks and then evenly mixed and sampled to obtain a representative sample from all areas of propagation. A representative sample was then transported to Jomo Kenyatta university's food science laboratories for processing. The sample was oven-dried for 48 hours at 60^0C and ground to pass through a 1mm sieve. A representative sample was submitted to Kenya Plant Health Inspectorate Service (KEPHIS) for phytosanitary certification. Ensuing certification, the sample was packaged aptly in aluminum bags and consigned to Obihiro university, Japan.

3.4.2 Rumen fluid collection and Cows' management

Rumen fluid for the *in vitro* study was obtained from two non-lactating fistulated Friesian cows which had an average weight and age of 709 kg BW and 10 years respectively. They were maintained on a basal diet of mixture of grasses namely; Timothy grass (*Phleum pretense)*, orchard grass (*dactylis*), crabgrass (*digitaria*), and fountain grass (*Pennisetum setaceum*) which contained DM, 958 g/kg; OM, 900 g/kg; Ash, 100 g/kg; CP, 99.3 g/kg; NDF, 542 g/kg; ADF,

327 g/kg; Lignin, 22.7 g/kg and EE, 33.3 g/kg on DM basis. The cows had an unlimited access to lick mineral block during the research period. The mineral block had the following mineral content per kilogram; yellow iron oxide (1742mg), iron sesquioxide (196mg), copper sulfate (377mg), cobalt sulfate (66mg), zinc sulfate (1235mg), manganese carbonate (1046mg), calcium iodate (77mg), sodium selenite (33mg) and common salt (971 g). Water was provided to the cows *ad libitum*. Reservation and sanction to use the two fistulated cows as a source of rumen was done by the university animal use, ethics and welfare committee ensuing timely application for the same. Following use of fistulated cattle approval, inception of feed acclimatization started ten days prior the commencement of rumen fluid collection. During the acclimatization period, the animals were fed twice daily, early in the morning (8: 00 am) and evening (4: 00 pm). The rumen fluid was collected from both cows, in each collection, they donated an equal amount of rumen fluid (about half a liter from each cow). Rumen fluid collection was done early in the morning three hours post-feeding, where the collection was effected by use of vacuum pump that would aspirate rumen liquor from the rumen. The collected rumen fluid would then be strained through a muslin cloth to filter the debris and immediately emptied into pre-warmed thermos flasks; the flasks were pre-warmed by use of tepid water maintained at 39^0C. The collected rumen fluid contained in the two pre-warmed thermos flasks would then be transported forthwith to Obihiro University's environmental hygiene laboratory for *in vitro* experiments and digestibility tests.

3.4.3 *In vitro* fermentation and gas production

Here, four treatments were formulated, and consisted the following; control (T1, 6 g Kikuyu grass sample + 4 g concentrate), treatment 2 (T2, 6 g of Kikuyu grass + 3 g of concentrate + 1 g of Euglena), treatment 3 (T3, 6 g of Kikuyu grass + 2.5 g of concentrate + 1.5 g of Euglena), treatment 4 (T4, 6 g of Kikuyu grass +2 g of concentrate + 2 g of Euglena). The samples were assigned and incubated in either of the four fermentation jars of the continuous substrate fermentation and continual gas quantification that was previously delineated by Sar *et al.,* (2005) randomly for the first running but in subsequent replications, it was done by following a systematic order. The fermentation cultures included the buffer solution and rumen liquor; the former was prepared according to McDougall *et al.,*(1948), autoclaved for sterilization at 121^0C for 20 minutes and CO_2 was flushed into it continuously for one hour while the latter was strained before being dispensed into each fermentation vessel proportionally. 80 milliliters from each cow was emptied into each fermentation jar making a total of 160 milliliters of rumen liquor. The ratio of buffer solution to rumen liquor was 4:1; that is, 640 ml of the buffer solution and 160 ml of the rumen fluid, totaling to 800 ml of fermentation cultures. The fermentation process was allowed to take place for 24 hours at 39^0 C under continuous flow of nitrogen gas at the rate of 20-25ml/min and an uninterrupted stirring at 33 rpm. pH and Oxidation Reduction Potential (ORP) sensors were inserted into each fermentation jar and screwed to measure the respective parameters and transmit the data to the analyzer and PC at one-minute interval.The gas production from each fermentation jar was measured at 30 minutes interval for 10 minutes and recorded continuously by a PC software. After completion of the fermentation process, 10 ml of incubation medium samples were collected and stored for VFA and ammonia nitrogen (NH_3-N) at -20^0 C. 1ml of rumen fluid from each jar was mixed with 4 ml of methylgreen-

formalin-saline in 10 ml tubes, wrapped in aluminum foil and stored in a dark chamber awaiting ciliates counting (Ogimoto and Imai, 1981). At the end of the 24th hour, the fermentation system would be stopped, fermentation medium discharged aptly and fermentation vessels washed properly and autoclaved in preparation for the subsequent fermentation period. CH_4 data from PC would then be retrieved after every round of fermentation.

3.4.4 Measurement of CH_4, NH_3-N analysis, and protozoa counting

CH_4 production in each fermentation jar was continually measured by near-infrared analyzer that was fixed in the continuous fermentation and continual gas quantification system and recorded by a PC software. The whole fermentation period data would then be retrieved from the PC, the data sorted and arranged according to the treatments. CH_4 production was quantified by multiplying nitrogen gas flow rate by CH_4 concentration for each recording then totaled cumulatively. The samples for NH_3-N analysis were thawed prior the experiment and the analysis was conducted according to the procedures outlined by Conway and O'Malley, (1942). Immediately after fermentation medium sample collection, 1ml of the sample was mixed with 4 ml of Methylgreen-Formalin-Saline (MFS) solution, wrapped in aluminum paper for ciliates counting. The ciliates were counted according to the procedures described by Ogimoto and Imai, (1981).

3.4.5 Analysis of volatile fatty acids

Total Volatile Fatty Acids (TVFA) and individual VFA were determined by use of GC-2010 (Shimadzu corporation) which had somewhat similar operating features as those described by Jaroslav and Rudolf, (2009). Samples and standards were prepared as outlined by Joaquin *et al.,* (2015) but with slight modification. The Appendix below explicitly outlines the procedures followed to prepare the samples and the standards for VFA analysis. The samples were prepared and ran in duplicates; 32 and 4 standards were analyzed.

3.4.6 *In vitro* digestibility of nutrients

This experiment involved incubation of 0.5 g of the samples in the strained rumen fluid; this was in accordance with the first step of the two-stage technique described by Tilley and Terry, (1963). The treatments were the following; treatment 1 (control, 0.3 g of kikuyu grass and 0.2 g of concentrate), treatment 2 (0.3 g of kikuyu grass + 0.150 g of concentrate + 0.05 g of Euglena), treatment 3 (0.3 g of kikuyu grass + 0.125 g of concentrate + 0.075 g of Euglena) and treatment 4 (0.3 g of kikuyu grass + 0.1 g of concentrate + 0.1 g of Euglena). The weighed samples were then put into 300 ml plastic bottles, into which 40 ml of McDougall's buffer was added. The

bottles were pre-warmed at 39^0C prior to addition of 10 ml of strained rumen fluid and were then sealed under a constant supply of CO_2 gas. The mixture of fermentation mediums and feed substrates was incubated at 39^0C for 24 hours. During the incubation period, the bottles were cautiously and gently swirled intermittently to obviate settling of the substrates and to allow even distribution of the substrate in the medium. After 24 hours, the bottle content was sieved through pre-weighed Gooch crucibles to obtain the bottle residue. Then, the residual DM was determined and the discrepancy in weight was regarded as an IVDMD. The residual content of IVDMD was ashed to determine IVOMD.

3.5 Laboratory analyses

The experimental samples were aptly prepared in readiness for laboratory analyses. AOAC, (1990) procedures were used to determine DM content by following procedure 930.15 of AOAC, (1990) which involved drying of 1g of the sample at 135^0C for two hours in the oven. N was determined by use of Kjeldahl method outlined by the procedure 984.13; CP was calculated by multiplying resultant N by 6.25. EE was determined according to a slightly modified procedure 920.29. Total ash was determined according to the guidelines outlined by the procedure 942.05 0f AOAC, (1990). The concentration of ADF and NDF was determined by strictly following procedure 973.18 of AOAC, (1990) and Van Soest *et al,* (1991) respectively with the exclusion of amylase usage in NDF determination and both expressed inclusive of residual ash. The concentration of lignin in the experimental samples was determined by solubilizing cellulose with sulfuric acid leaving lignin intact for measurement. Gross Energy(GE) was determined by combusting the experimental samples in a bomb calorimeter. The composition of amino acids of both Euglena and kikuyu grass was analyzed by Japan Food Research Laboratories, Japan. An automatic amino acid analysis method was used to analyze all the other amino acids except tryptophan which was analyzed by use of a high-performance liquid chromatography. Fatty acids composition of the aforementioned experimental substrates was analyzed by Gas chromatography, GC-1700, Shimadzu corporation, Japan. The Volatile Fatty Acids (VFAs) composition of the collected fermentation medium samples was analyzed by gas chromatography, GC-2010.

3.6 Statistical analyses

The data from *in vivo* and *in vitro* experiments were sorted, entered into a computer spreadsheet and descriptive statistical analysis was done. An inferential statistical analysis was conducted by use of polynomial regression analysis (Proc REG) (SAS, 2010). Polynomial regression was conducted at all levels; that is, linear, quadratic, and cubic and the trend of the effects evaluated. The difference amongst the means was determined by use of Turkey's Honesty Significant Difference test (Turkey's HSD) option of SAS, (2010). A statistical significance threshold of factors of investigation was set at $p< 0.05$. The standard error of means (SEM) was obtained by use of least squares means in ISmeans of SAS Institute, (2010).

4 Results

4.1 Chemical composition of experimental substrates

Chemical composition determination of all experimental substrates was conducted, and the results presented in the Table 4.1.1. Table 4.1.2 shows the resultant chemical composition of the experimental diet ensuing different levels of Euglena inclusion into the diet.

Table 4.1.1 Chemical Composition of all the experimental substrates and supplements

parameters	Klein grass	kikuyu grass	Concentrate	Euglena (*in vivo*)	Euglena (*in vitro*)
DM (g/kg)	955	936	951	969	950
Ash (g/kg DM)	84.7	116.0	71.7	35.9	75.5
OM (g/kg DM)	915	884	928	964	924
CP (g/kg DM)	101.3	81.8	182.3	285	346
NDF (g/kg DM)	650	614	232	6.5	1.7
ADF (g/kg DM)	368	305	37.5	2.8	0.2
Lignin (g/kg DM)	20.3	29.7	7.5	0.8	0.2
EE (g/kg DM)	21.1	21.2	36.3	132.2	153.0
GE (MJ/kg DM)	17.5	16.4	17.8	21.4	

DM: Dry Matter ADF: Acid Detergent Fiber CP: Crude Protein
OM: Organic Matter NDF: Neutral Detergent Fiber
EE: Ether Extract GE: Gross Energy

Table 4.1.2 Chemical composition of the experimental diet after various levels of Euglena inclusion

	***in vivo* study**				***in vitro* study**			
	T1	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**
Parameters	**0**	**5%**	**10%**	**15%**	**0%**	**10%**	**15%**	**20%**
DM (g/kg)	953	954	955	956	942	942	942	942
Ash (g/kg DM)	79.5	77.7	76.6	75.2	98.3	98.7	98.9	99.0
OM (g/kg DM)	921	922	923	925	902	901	901	901
CP (g/kg DM)	134	139	140	143	122	138	147	155
NDF (g/kg DM)	483	472	460	449	461	438	427	415
ADF (g/kg DM)	236	234	232	231	216	208	204	200
Lignin (g/kg/DM)	15.2	14.8	15.1	15.2	20.8	20.1	19.7	19.4
EE (g/kg DM)	27.2	32.0	36.1	40.4	27.2	38.9	44.7	50.6

DM: Dry matter ADF: Acid Detergent Fiber
OM: Organic Matter NDF: Neutral Detergent Fiber
CP: Crude protein EE: Ether extract

Table 4.1.3 shows the amino and fatty acids content of kikuyu grass which was used as an experimental substrate in the *in vitro* study.

Table 4.1.3 Amino and Fatty acids of Kikuyu grass

Amino acids (AA)	g/100g AA	g/100g of the grass	Fatty acids (FA)	g/100g FA	g/100g of the grass
Arg	4.79	0.30	C12:0	0.7	0.03
Lys	5.30	0.33	C14:0	0.8	0.03
His	2.26	0.14	C15:0	0.3	0.01
Phe	5.16	0.32	C16:0	22.4	0.81
Tyr	2.89	0.18	C16:1	1.2	0.04
Leu	7.58	0.47	C17:0	1.2	0.04
Ile	3.97	0.25	C18:0	2.0	0.07
Met	1.68	0.10	C18:1	4.2	0.15
Val	5.78	0.36	18:2n-6	18.7	0.68
Ala	6.87	0.43	18:3n-3	36.2	1.31
Gly	5.30	0.33	C20:0	1.0	0.04
Pro	8.48	0.53	20:3n-6	0.2	0.01
Glu	11.8	0.73	C22:0	1.0	0.04
Ser	5.80	0.36	C22.1	0.3	0.01
Thr	4.68	0.29	C24:0	1.6	0.06
Asp	14.10	0.88	SFA	31.0	1.12
Trp	1.92	0.12	MUFA	5.7	0.21
Cys	1.65	0.10	PUFA	55.1	2.00

Arg: Arginine Leu: Leucine Gly: Glycine Asp: Aspartic acid
Lys: Lysine ile: isoleucine Pro: Proline Trp: Tryptophan
His: Histidine Met: methionine Glu: Glutamic acid Cys: Cysteine
Phe: Phenylalanine Val: Valine Ser: Serine
Try: Tryptophan Ala: Alanine Thr: Threonine
SFA: Saturated Fatty Acids
MUFA: Monounsaturated Fatty Acids.
PUFA: Polyunsaturated Fatty Acids.

Table 4.1.4 and Table 4.1.5 show amino and fatty acids profile of Euglena used in both studies, that is, *in vivo* and *in vitro* studies.

Table 4.1.4 Amino acids content of Euglena used in *in vivo* and *in vitro* studies

Amino acids of Euglena (*in vitro*)			Amino acids of Euglena (*in vivo*)		
Amino acids	g/100g Euglena	g/100g Amino Acids	Amino acids	g/100g Euglena	g/100g Amino Acids
Arg	2.05	6.96	Arg	1.97	6.94
Lys	2.14	7.27	Lys	2.06	7.25
His	0.80	2.72	His	0.79	2.78
Phe	1.37	4.66	Phe	1.34	4.72
Tyr	1.22	4.15	Tyr	0.98	3.45
Leu	2.48	8.43	Leu	2.44	8.59
Ile	1.19	4.04	Ile	1.17	4.12
Met	0.67	2.28	Met	0.68	2.39
Val	1.94	6.59	Val	1.92	6.76
Ala	2.35	7.99	Ala	2.21	7.78
Gly	1.53	5.20	Gly	1.47	5.18
Pro	1.88	6.39	Pro	1.73	6.09
Glu	3.48	11.82	Glu	3.45	12.15
Ser	1.26	4.28	Ser	1.22	4.29
Thr	1.48	5.03	Thr	1.44	5.07
Asp	2.53	8.60	Asp	2.54	8.94
Trp	0.52	1.77	Trp	0.50	1.76
Cys	0.54	1.83	Cys	0.49	1.73

Arg: Arginine	Leu: Leucine	Gly: Glycine	Asp: Aspartic acid
Lys: Lysine	ile: isoleucine	Pro: Proline	Trp: Tryptophan
His: Histidine	Met: methionine	Glu: Glutamic acid	Cys: Cysteine
Phe: Phenylalanine	Val: Valine	Ser: Serine	
Try: Tryptophan	Ala: Alanine	Thr: Threonine	

Table 4.1.5 Fatty acids content of Euglena used in both studies

Fatty Acids content of Euglena used in both studies					
Fatty acids of Euglena (*in vitro*)			Fatty acids of Euglena (*in vivo*)		
Fatty acids	(g/100g lipid)	g/100g Euglena	Fatty acids	(g/100g lipid)	g/100g Euglena
C10:0	0.2	0.033	C12:0	2.1	0.292
C12:0	3.7	0.614	C13:0	5.3	0.737
C13:0	7.3	1.213	C14:0	28.1	3.906
C14:0	31.0	5.149	C15:0	2.3	0.319
C14:1	0.3	0.049	C16:0	11.2	1.557
C15:0	2.9	0.482	C16:1	2.5	0.348
C16:0	10.6	1.761	C17:0	0.7	0.097
C16:1	3.6	0.598	C17:1	1.2	0.167
C17:0	0.7	0.116	C18:0	2.9	0.403
C17:1	1.4	0.232	C18:1	7.4	1.029
C18:0	1.8	0.299	18:2n-6	3.6	0.500
C18:1	5.3	0.880	18:3n-3	1.4	0.195
C18:2n-6	2.4	0.399	C20:0	0.2	0.028
C18:3n-3	1.0	0.166	20:2n-6	3.8	0.528
C20:1	0.3	0.049	20:3n-6	6.4	0.889
C20:2n-6	2.6	0.431	20:3n-3	0.4	0.055
C20:3n-6	4.6	0.764	20:4n-6	6.3	0.876
C20:3n-3	0.3	0.049	20:4n-3	1.5	0.209
C20:4n-6	5.4	0.897	20:5n-3	1.3	0.181
C20:4n-3	1.1	0.183	22:4n-6	3.9	0.542
C20:5n-3	1.0	0.166	22:5n-6	1.9	0.264
C20:4n-6	3.2	0.532	225n-3	0.4	0.056
C20:5n-6	1.2	0.199	22:6n-3	0.1	0.014
C20:5n-3	0.2	0.033	C24:0	0.2	0.028
SFA	58.2	9.670	**SFA**	53.0	7.368
MUFA	10.9	1.810	**MUFA**	11.1	1.543
PUFA	23.0	3.820	**PUFA**	31.0	4.309

SFA: Saturated Fatty Acids
MUFA: Monounsaturated Fatty Acids.
PUFA: Polyunsaturated Fatty Acids.

4.2 In vivo

4.2.1 Effects of Euglena supplementation on nutrients utilization.

Euglena supplementation had some notable effects on nutrients intake as revealed by results. DM intake ranged from 710 to 757 g/day. The increment of Euglena inclusion in the basal diet from zero percent inclusion in T1 to fifteen percent inclusion in T4 exhibited both linear ($p<0.01$) and quadratic ($p<0.05$) effects on DM intake. The inclusion of Euglena in the basal diet depicted both linear ($p<0.01$) and quadratic ($p<0.05$) effects on all other nutrients (CP, OM, ADF and GE) except NDF intake which showed only quadratic ($p<0.05$) effect. CP and GE intake increased both linearly ($p<0.01$) and quadratically ($p<0.05$) as shown in the Table 4.2.1.

Table 4.2.1 Effects of Euglena supplementation on nutrients intake

	Treatments					P-values		
Item	T1	T2	T3	T4	SEM	L	Q	C
DM (g/d)	710^{b}	742^{a}	761^{a}	757^{a}	2.393	0.0001	0.0021	0.6521
CP (g/d)	96.7^{c}	105.0^{b}	110.0^{ab}	114.0^{a}	0.767	0.0001	0.0011	0.9552
OM (g/d)	653^{c}	684^{b}	703^{a}	701^{ab}	2.592	0.0001	0.0021	0.6484
NDF(g/d)	344^{ab}	351^{a}	351^{a}	341^{b}	1.126	0.5093	0.0012	0.7082
ADF(g/d)	168^{b}	176^{a}	180^{a}	179^{a}	0.566	0.0001	0.0019	0.6218
GE (MJ/d)	12.5^{c}	13.2^{b}	13.7^{a}	13.8^{a}	0.043	0.0001	0.0019	0.6305

DM: Dry matter ADF: Acid Detergent Fiber
CP: Crude Protein NDF: Neutral Detergent Fiber
OM: Organic Matter GE: Gross Energy
$^{a-c}$: Different superscripts show the statistical difference among the means within the same row

Generally, Euglena supplementation did not exhibit a substantial effect on nutrients digestibility. Euglena supplementation exhibited a linear ($p<0.05$) effect on both CP and GE digestibility. On all the other four nutrients (DM, OM, NDF and ADF) there were no observed supplementation effects of Euglena inclusion in the basal diet (P>0.05) as shown in the Table 4.2.2.

Table 4.2.2 *In vivo* effects of Euglena supplementation on nutrients digestibility

	Treatments				SEM	*P-values*		
Parameters	T1	T2	T3	T4		L	Q	C
DM	0.74	0.73	0.73	0.74	0.005	0.8321	0.3074	0.8472
CP	0.69^{b}	0.69^{ab}	0.71^{ab}	0.72^{a}	0.005	0.0094	0.4851	0.8511
OM	0.75	0.74	0.74	0.76	0.005	0.8932	0.2324	0.7659
NDF	0.70	0.67	0.66	0.67	0.006	0.157	0.1886	0.6344
ADF	0.63	0.61	0.58	0.62	0.078	0.5701	0.1047	0.2443
GE	0.81^{a}	0.79^{ab}	0.72^{c}	0.73^{bc}	0.011	0.0006	0.2616	0.0656

DM: Dry matter ADF: Acid Detergent Fiber
CP: Crude Protein NDF: Neutral Detergent Fiber
OM: Organic Matter GE: Gross Energy
$^{a-c}$: Different superscripts show the statistical difference among the means within the same row

Euglena supplementation showed linear effects on nutrients (DM, CP, OM, and DE) digested ($p<0.05$). The amount of CP digested increased linearly ($p<0.01$) as Euglena inclusion percentage in the diet increased. The amount of CP digested increased from as low as 66 g/day in T1, the non-supplemented one, to 83 g/day in T4, the 15 percent Euglena supplemented one. Euglena supplementation did not have any effect ($p>0.05$) on the other remaining nutrients (DM, NDF, and ADF) as shown in the Table 4.2.3.

Table 4.2.3 *In vivo* effects of Euglena supplementation on nutrients digested

	Treatments				SEM	*P-values*		
Parameters	T1	T2	T3	T4		L	Q	C
DM (g/day)	521^{b}	541^{ab}	553^{ab}	564^{a}	2.393	0.0001	0.6114	0.8334
CP (g/day)	66^{c}	72^{ab}	78^{ab}	83^{a}	0.765	0.0001	0.6632	0.9132
OM (g/day)	490^{b}	509^{ab}	520^{ab}	532^{a}	3.241	0.0001	0.6643	0.7641
NDF (g/day)	238	237	231	231	2.034	0.1701	0.9304	0.5778
ADF (g/day)	105	108	105	113	1.287	0.1307	0.4142	0.1931
DE (MJ/d)	6.6^{c}	7.0^{b}	6.8^{bc}	7.4^{a}	0.096	0.0191	0.5973	0.1002

DM: Dry matter ADF: Acid Detergent Fiber
CP: Crude Protein NDF: Neutral Detergent Fiber
OM: Organic Matter DE: Digestible Energy
$^{a-c}$: Different superscripts show the statistical difference among the means within the same row

Table 4.2.4 The combined effects of Euglena supplementation on nutrients intake, digested, and digestibility.

	Treatments				SEM	*P-values*		
parameters	T1	T2	T3	T4		L	Q	C
DM Intake	710^{b}	742^{a}	761^{a}	757^{a}	2.393	0.0001	0.0021	0.6521
DM digested	521^{b}	541^{ab}	553^{ab}	564^{a}	2.393	0.0001	0.6114	0.8334
DM digestibility	0.74	0.73	0.73	0.74	0.005	0.8321	0.3074	0.8472
CP intake	96.7^{c}	105.0^{b}	110.0^{ab}	114.0^{a}	0.767	0.0001	0.0011	0.9552
CP digested	66^{c}	72^{ab}	78^{ab}	83^{a}	0.765	0.0001	0.6632	0.9132
CP digestibility	0.69^{b}	0.69^{ab}	0.71^{ab}	0.72^{a}	0.005	0.0094	0.4851	0.8511
OM intake	653^{c}	684^{b}	703^{a}	701^{ab}	2.592	0.0001	0.0021	0.6484
OM digested	490^{b}	509^{ab}	520^{ab}	532^{a}	3.241	0.0001	0.6643	0.7641
OM digestibility	0.75	0.74	0.74	0.76	0.005	0.8932	0.2324	0.7659
NDF Intake	344^{ab}	351^{a}	351^{a}	341^{b}	1.126	0.5093	0.0012	0.7082
NDF digested	238	237	231	231	2.034	0.1701	0.9304	0.5778
NDF digestibility	0.70	0.67	0.66	0.67	0.006	0.157	0.1886	0.6344
ADF Intake	168^{b}	176^{a}	180^{a}	179^{a}	0.566	0.0001	0.0019	0.6218
ADF digested	105	108	105	113	1.287	0.1307	0.4142	0.1931
ADF digestibility	0.63	0.61	0.58	0.62	0.078	0.5701	0.1047	0.2443
GE intake	12.5^{c}	13.2^{b}	13.7^{a}	13.8^{a}	0.043	0.0001	0.0019	0.6305
Digestible energy	6.6^{c}	7.0^{b}	6.8^{bc}	7.4^{a}	0.096	0.0191	0.5973	0.1002
GE digestibility	0.81^{a}	0.79^{ab}	0.72^{c}	0.73^{bc}	0.011	0.0006	0.2616	0.0656

DM: Dry matter ADF: Acid Detergent Fiber
CP: Crude Protein NDF: Neutral Detergent Fiber
OM: Organic Matter GE: Gross Energy
$^{a-c}$: The means within the same row with different superscripts are statistically different

Euglena supplementation had some intriguing effects on CP intake, digestion, retention, and loss. As earlier stated, CP intake increased both linearly ($p<0.01$) and quadratically ($p<0.05$). CP digested and digestibility depicted a linear ($p<0.05$) increment. Total CP loss increased both linearly ($p<0.01$) and quadratically ($p<0.05$). The supplementation had both linear ($p<0.01$) and quadratic ($p<0.05$) impacts on urinary CP loss while it did not have any significant effect on fecal CP loss ($p>0.05$). On the other hand, Euglena supplementation had statistically significant effect on CP retained; CP retained showed both linear ($p<0.05$) and quadratic ($p<0.05$) increments. Table 4.2.5 shows various supplementation effects on Euglena.

Table 4.2.5 Effects of Euglena supplementation on CP intake, digested, and digestibility

	Treatments				**SEM**	***P-values***		
parameters	T1	T2	T3	T4		L	Q	C
CP intake	96.7^{d}	105.0^{c}	110.0^{b}	114.0^{a}	0.24	0.0001	0.0003	0.9094
CP digested	66^{c}	72^{ab}	78^{ab}	83^{a}	0.96	0.0001	0.4330	0.8482
CP digestibility	0.69^{b}	0.69^{ab}	0.71^{ab}	0.72^{a}	0.92	0.0122	0.8627	0.9317
Urinary CP loss	38.4^{c}	44.5^{b}	51.2^{a}	46.5^{ab}	0.74	0.0001	0.0020	0.0703
Fecal CP loss	20.9	21.7	21.3	20.1	0.71	0.6785	0.3063	0.9718
Total CP loss	59.3^{b}	66.2^{ab}	72.5^{a}	66.6^{a}	0.88	0.0006	0.0021	0.1397
CP retained	32.6^{b}	34.3^{b}	34.2^{b}	42.9^{a}	0.69	0.0003	0.0171	0.0687

CP: Crude protein
$^{a-d}$: The means within the same row with different superscripts differ statistically

Euglena supplementation had varied effects on urinary and fecal CP loss and CP digested in relation to CP and DM intake and total CP loss. For instance, the supplementation had a linear effect ($p<0.05$) on both fecal and urinary CP losses in relation to total CP loss. There was a substantial relation between CP retained and CP intake. There were both linear ($p<0.01$) and quadratic ($p<0.01$) effects on CP retained in relation to CP intake. CP intake in relation to DM intake increased linearly (P<0.05) along the treatments. Euglena inclusion in the basal diet showed both linear and quadratic ($p<0.05$) effects on urinary CP loss in relation to CP intake but did not have any statistically significant effect ($p>0.05$) on fecal CP loss in relation to CP intake. Finally, the supplementation had both linear and quadratic ($p<0.05$) impacts on total CP loss in relation to total CP intake. Table 4.2.6 shows various intriguing effects on CP metabolism.

Table 4.2.6 The metabolism of CP ensuing Euglena supplementation

	Treatments				SEM	*P-values*		
parameters	T1	T2	T3	T4		L	Q	C
Urinary CP loss /total CP loss	0.65^{b}	0.67^{ab}	0.71^{a}	0.70^{a}	0.005	0.0137	0.1851	0.3495
Fecal CP loss /Total CP loss	0.35^{a}	0.33^{ab}	0.29^{b}	0.30^{b}	0.875	0.0137	0.1851	0.3495
CP retained /CP intake	0.33^{b}	0.31^{b}	0.31^{b}	0.38^{a}	0.004	0.0001	0.0001	0.4379
CP intake /DM intake	0.14^{b}	0.14^{b}	0.15^{ab}	0.15^{a}	0.177	0.0046	0.8442	0.9114
Urinary CP loss /CP intake	0.40^{b}	0.43^{ab}	0.46^{a}	0.41^{ab}	0.008	0.0316	0.0149	0.1436
fecal CP loss /CP intake	0.22	0.21	0.19	0.18	0.767	0.0622	0.7256	0.9577
total CP loss /CP intake	0.61^{ab}	0.63^{ab}	0.66^{a}	0.59^{b}	0.007	0.0068	0.0031	0.1003

CP: Crude protein
DM: Dry Matter
$^{a-b}$: The means within the same row with different superscripts differ statistically.

Euglena supplementation showed manifold effects on energy intake, digestibility, and losses. There were both linear ($p<0.05$) and quadratic ($p<0.05$) increase in GE intake (MJ/d) among the treatments. GE digestibility depicted a linear ($p<0.05$) effect ensuing Euglena supplementation, this was also similar for DE intake (MJ/d) which exhibited a linear ($p<0.01$) response to Euglena supplementation. However, the supplementation did not have any statistically significant effect on ME (MJ/d). Euglena supplementation did not show any impact on Urinary Energy Loss (UEL) but had statistically significant effect on Fecal Energy Loss (FEL). There were linear, cubic ($p<0.05$) and quadratic ($p<0.05$) effects on FEL (MJ/D). There was no statistically significant effect on total energy loss ($p>0.05$); this Total Energy Loss (TEL) did not include gaseous energy loss but it was a sum of urinary and fecal energy losses. Euglena supplementation did not have any significant impact on both UEL and FEL (MJ/d) in relation to TEL ($p>0.05$), but on the other hand, it had a significant impact on TEL (MJ/d) in relation to GE intake. There were statistically significant effects on TEL/GE intake at all levels of polynomial regression analysis, that is, linearly, cubically ($p<0.01$) and quadratically ($p<0.05$). Finally, Euglena supplementation had a significant impact on FEL (MJ/d) in relation to GE intake at all the levels of polynomial analysis depicting linear and cubic ($p<0.01$) and quadratic ($p<0.05$) impacts. Table 4.2.7 shows Euglena supplementation effects on energy intake, digestibility, and losses.

Table 4.2.7 Effects of Euglena supplementation on energy intake, digestibility, and losses

Parameters	Treatments T1	T2	T3	T4	SEM	*P-values* L	Q	C
GE intake(MJ/d)	12.5[c]	13.2[b]	13.7[a]	13.8[a]	0.065	0.0001	0.0019	0.6305
GE digestibility	0.8[a]	0.8[ab]	0.7[c]	0.7[bc]	0.011	0.0006	0.2616	0.0656
DE intake	6.6[c]	7.0[b]	6.8[bc]	7.4[a]	0.068	0.0001	0.3721	0.0014
ME intake	8.5	8.2	7.7	6.8	0.537	0.3612	0.6588	0.9532
UEL	0.4	0.5	0.5	0.4	0.017	0.2002	0.0431	0.9071
FEL(MJ/d)	5.9[c]	6.2[b]	6.9[a]	6.3[b]	0.044	0.0001	0.0015	0.0001
TEL	6.3	6.7	7.4	6.7	0.568	0.7328	0.788	0.6837
TEL/GE intake	0.50[b]	0.51[b]	0.54[a]	0.49[b]	0.345	0.0001	0.0125	0.0001
UEL/TEL	0.06	0.07	0.07	0.06	0.248	0.6245	0.5084	0.2961
FEL/TEL	0.94	0.93	0.94	0.94	0.248	0.6245	0.5084	0.2961
FEL/GE intake	0.47[b]	0.46[b]	0.50[a]	0.46[b]	0.359	0.0001	0.0259	0.0001
UEL/GE intake	0.03	0.04	0.04	0.03	0.082	0.4724	0.1304	0.9959

GE: Gross Energy UEL: Urinary Energy Loss

DE: Digestible Energy FEL: Fecal Energy Loss

ME: Metabolizable Energy TEL: Total Energy Loss

[a-c]: The means within the same row with different superscripts differ statistically

4.2.2 Effects of Euglena supplementation on rumen fermentation parameters

Table 4.2.8 Overall effects of Euglena supplementation on rumen fermentation parameters

	Treatment				SEM	*P-values*		
Rumen parameters	T1	T2	T3	T4		L	Q	C
Protozoa (cell/ml × 10^6)	3.0^a	2.8^b	1.5^c	1.1^d	0.040	0.0001	0.3390	0.0001
NH_3-N (mg/L)	41.8^b	42.8^b	61.8^a	66.2^a	1.818	0.0001	0.0028	0.0591
PH	6.7	6.6	6.5	6.7	0.042	0.3362	0.0233	0.2765
TVFA (mol/100ml)	55.6	57.8	62.2	57.5	2.091	0.7251	0.7865	0.9846

NH_3-N: Ammonia Nitrogen **TVFA**: Total Volatile Fatty Acids
$^{a-d}$: The means within the same row with different superscripts are statistically different

Table 4.2.8 shows Euglena supplementation effects on overall rumen fermentation parameters. It is explicit that Euglena inclusion in the diet had pronounced impacts on overall protozoa cells per milliliter of rumen fluid. The ciliate counts in rumen fluid reduced significantly ensuing increment of Euglena concentration in the diet. It depicted linear and cubic ($p<0.001$) decline as Euglena content in the experimental diet increased. Overall NH_3-N concentration in rumen fluid tended to significantly increase as Euglena concentration in the diet increased. There was no significant influence on overall rumen pH values ensuing Euglena supplementation. Additionally, there were no significant impacts on Total Volatile fatty Acids (TVFA).

4.2.2.1 pH

The inclusion of Euglena in the diet did not have any effect ($p>0.05$) on the overall ruminal pH values which averaged out at 6.63. However, there were intriguing post-feeding effects of Euglena inclusion on ruminal pH. For the first eight hours post-feeding, there was intermittent Euglena inclusion effect on ruminal pH with 0 hour after feeding recording both linear and quadratic ($p<0.05$) effects while in the 2nd-hour post feeding, there was no significant effect on ruminal pH. At the 4th-hour post feeding, there was a significant effect on pH observed at all levels of polynomial regression analysis ($p<0.05$) while at the 6th hour, there was no statistical significance among the treatments. At the 8th hour, there was a significant linear ($p<0.05$) ruminal pH decline with the increment of Euglena inclusion percentage in the diet. However, at 24th-hour post feeding, there was no significant effect ($p>0.05$) on ruminal pH among the treatments but there was a notable pH increment in all treatment which averaged out at 7.17 from previous collection hour whose average in all the treatments was 6.42. Table 4.2.9 shows the effects of Euglena inclusion on ruminal pH.

Table 4.2.9 Effects of Euglena supplementation on pH

	Treatments				SEM	*P-values*		
Hours post-feeding	T1	T2	T3	T4		L	Q	C
0 hour	6.87[a]	6.62[b]	6.61[b]	6.87[a]	0.030	0.004	0.002	0.329
2 hours	6.61	6.48	6.33	6.61	0.060	0.081	0.023	0.238
4 hours	6.65[a]	6.52[a]	6.16[b]	6.56[a]	0.060	0.003	0.016	0.013
6 hours	6.62	6.52	6.36	6.48	0.060	0.189	0.184	0.347
8 hours	6.59[a]	6.48[ab]	6.37[ab]	6.27[b]	0.048	0.023	0.937	0.959
24 hours	7.13	7.08	7.22	7.23	0.013	0.005	0.356	0.008

[a-b]: The means within the same row with different superscripts are statistically different

4.2.2.2 Ciliate protozoa count

Euglena supplementation had a pronounced impact on ciliate protozoa count. Microscopic protozoa enumeration revealed both linear and cubic ($p<0.01$) decline among the treatments as Euglena supplementation level increased.

4.2.2.3 Ruminal ammonia nitrogen (NH_3-N) concentration

The overall ruminal NH_3-N concentration increased among the treatments as Euglena concentration in the diet increased. Increasing Euglena inclusion percentage resulted in linear ($p<0.01$) and quadratic ($p<0.05$) effects on ruminal NH_3-N concentration.

Euglena inclusion in the basal diet had a significant impact on ruminal NH_3-N concentration. Immediately after morning feeding, a high ruminal NH_3-N concentration was recorded which tended to gradually dwindle as time elapsed during the day in all the treatments. Across the treatments, there was initial linear, quadratic and cubic ($p<0.05$) effects on ruminal NH_3-N concentration as Euglena concentration increased in the diet. Two hours later, the same effect impacted by Euglena supplementation persisted except that there was no cubic effect ($p=0.068$) but there was a notable difference in ruminal NH_3-N concentration between control and supplemented diets. At the 4^{th}, 6^{th} and 8^{th} hour, the ruminal NH_3-N concentration depicted both linear and cubic effects ($p<0.05$). In all the aforementioned hours, the average ruminal NH_3-N concentration in all the treatments was somewhat in close range. At the 24^{th} hour, the ruminal NH_3-N concentration increased as Euglena supplementation level in the diet increased, from 34.2 mg/ml in T1 to 66.2 mg/ml in T4. The increment was both linear ($p<0.01$) and cubic ($P<0.05$).

Table 4.2.10 Effects of Euglena supplementation on NH_3-N concentration in the rumen fluid

	Treatments				SEM	*P-values*		
	T1	T2	T3	T4		L	Q	C
0 hour	52.9^{c}	54.3^{a}	75.9^{ab}	74.9^{bc}	1.747	0.0003	0.0009	0.0083
2 hours	45.1^{b}	47.3^{a}	73.3^{a}	71.4^{a}	2.385	0.0005	0.0006	0.0677
4 hours	40.7^{b}	42.9^{b}	61.1^{a}	66.3^{b}	1.575	0.0008	0.7355	0.0002
6 hours	37.5^{b}	39.2^{b}	48.2^{a}	61.9^{a}	1.376	0.0002	0.5799	0.0147
8 hours	36.3^{ab}	38.1^{bc}	55.3^{a}	56.7^{c}	2.207	0.0005	0.1052	0.0002
24 hours	34.2^{b}	35.1^{b}	57.2^{a}	66.2^{a}	1.877	0.0001	0.547	0.0096

$^{a-c}$: The means within the same row with different superscripts are statistically different

4.2.2.4 Volatile fatty acids

Euglena supplementation did not have any substantial effect on TVFA concentration in the rumen fluid. The supplementation only had a significant effect on valeric which tended to have linear, quadratic and cubic ($p<0.05$) increment but recorded a slight decline in T4. Table 4.2.11 shows volatile fatty acids' data

Table 4.2.11 Effects of Euglena supplementation on volatile fatty acids

	Treatments					*P-values*		
parameters	T1	T2	T3	T4	SEM	L	Q	C
Acetate (mol/100ml)	30.1	29.9	32.1	30.2	0.659	0.2676	0.3451	0.1095
Propionic (mol/100ml)	12.7	12.8	12.7	12.9	0.252	0.8926	0.8414	0.6407
Butyric (mol/100ml)	12.9	13.0	14.7	12.5	0.526	0.0973	0.0885	0.0677
Valeric (mol/100ml)	1.7^{b}	1.8^{b}	2.4^{a}	1.7^{b}	0.087	0.0006	0.0025	0.0057
Caproic (mol/100ml)	0.2	0.3	0.3	0.2	0.021	0.0367	0.0065	0.3067
TVFA (mol/100ml)	57.6	57.8	62.2	57.5	1.269	0.1513	0.1525	0.0876
A/P (mol/100ml)	2.4	2.3	2.5	2.3	0.012	0.4467	0.4327	0.1635

TVFA: Total Volatile Fatty Acids
A/P: The ratio of acetic acids to propionic.

$^{a-b}$: Different superscripts show the statistical difference among the means within the same row

4.3 In vitro

4.3.1 Effects of Euglena inclusion in the diet on CH_4 emission and ORP

Euglena inclusion in the diet resulted in a decline in CH_4 emission in a dose-dependent manner, though the response was not statistically significant. Numerically, the reduction was by 35.4% after 20% inclusion level. Intriguingly, the rate of CH_4 reduction was gradual and even, at somewhat constant rate after every level of inclusion. Oxidation Reduction Potential (ORP) was not greatly affected by Euglena inclusion in the diet, albeit it tended to decrease with the increment of Euglena in the diet. However, the decline was not statistically significant. Table 4.3.1 shows the two parameters in the simulated ruminal conditions.

Table 4.3.1 Effects of Euglena on CH_4 emission and ORP

	Treatments				SEM	*P-values*		
Parameters	T1	T2	T3	T4		L	Q	C
ORP (mV)	-386.2	-398.1	-401.8	-405.1	5.40	0.6642	0.696	0.875
CH_4 (ml/24h)	195.2	165.9	148.9	126.1	9.47	0.142	0.8662	0.842

ORP: Oxidation Reduction Potential
CH_4: Methane

4.3.2 Effects of Euglena inclusion in the diet on simulated ruminal fermentation parameters

Euglena inclusion in the diet did not significantly affect protozoa population. However, the total protozoa count tended to slightly decline as Euglena inclusion in the diet increased. NH_3-N concentration in the fermentation medium significantly increased as Euglena content in the experimental diet increased. An increment by 67.3% was registered; NH_3-N concentration sharply increased after 10 % addition of Euglena into the experimental diet. pH was not affected at all by Euglena inclusion in the diet, it averaged out to 6.98±0.01. Table 4.3.2 shows the effect of Euglena on ruminal fermentation parameters. TVFA were not significantly affected by Euglena supplementation. Euglena inclusion in the experimental diet did not significantly affect specific VFA. The fermentation process favored formation of acetic over propionic, the production of the former was three times the latter. The ratio of Acetic (A) to Propionic (P) was also not significantly affected by Euglena addition into the experimental diet.

Table 4.3.2 Effects of Euglena supplementation on rumen fermentation parameters.

	Treatments				SEM	*P-values*		
Parameters	T1	T2	T3	T4		L	Q	C
Protozoa (cells/ml×10^3)	12.60	11.50	10.90	9.90	0.385	0.161	0.901	0.789
NH_3-N (mg/ml)[3]	7.84[b]	16.98[ab]	20.03[a]	23.94[a]	1.028	0.0002	0.272	0.515
TVFA (mol/100ml)	17.31	16.57	16.01	19.46	0.147	0.528	0.322	0.767
PH	6.99	6.98	6.97	6.96	0.025	0.982	1.000	0.851

NH_3-N: Ammonia nitrogen TVFA: Total Volatile Fatty Acids
[a-b]: Different superscripts show the statistical difference among the means within the same row.

Table 4.3.3 Effects of Euglena inclusion in the experimental diet on volatile fatty acids

	Treatments				*P-values*			
parameters	T1	T2	T3	T4	SEM	L	C	Q
TVFA (mol/100ml)	17.3	16.6	16.0	19.5	0.147	0.528	0.322	0.767
Acetic (mol/100ml)	11.7	11.2	10.8	13.1	0.023	0.561	0.354	0.558
Propionic (mol/100ml)	4.17	3.90	3.62	4.30	0.119	0.957	0.227	0.621
n-butyric (mol/100ml)	1.13	1.17	1.17	1.47	0.076	0.164	0.408	0.577
n-valeric (mol/100ml)	0.09	0.12	0.12	0.17	0.015	0.090	0.601	0.718
Isobutyric (mol/100ml)	0.19	0.20	0.20	0.27	0.022	0.322	0.621	0.778
Isovaleric (mol/100ml)	0.08	0.08	0.10	0.16	0.016	0.128	0.42	0.872
A:P (mol/100ml)	2.80	2.87	3.03	3.02	0.094	0.333	0.841	0.695

A:P: The ratio of acetate to propionate

4.3.3 Effects of Euglena on *in vitro* DM and OM digestibility

Euglena addition into the experimental diet significantly improved both *in vitro* DM digestibility (IVDMD) and *In vitro* OM digestibility (IVOMD). Table 4.3.4 shows the effect of Euglena on DM and OM digestibility in an *in vitro* setup.

Table 4.3.4 Effects of Euglena inclusion in the experimental diet on nutrients digestibility

	Treatments				SEM	*P-values*		
Parameters	T1	T2	T3	T4		L	Q	C
IVDMD	0.50^{c}	0.51^{bc}	0.54^{ab}	0.54^{a}	0.0032	0.0003	0.722	0.184
IVOMD	0.41^{c}	0.41^{bc}	0.44^{ba}	0.45^{a}	0.0034	0.0004	0.712	0.182

IVOMD: *In vitro* Organic Matter Digestibility
IVDMD: *In vitro* Dry Matter Digestibility
$^{a-b}$: Different superscripts show the statistical difference among the means within the same row

5 Discussion

5.1 *In vivo* study

5.1.1 Effects of Euglena supplementation on nutrients utilization

5.1.1.1 Nutrients intake

The increased level of Euglena inclusion in the diet resulted in a significant increment in DM intake up to 10% inclusion level, the increment was by 7.2% but at 15 % inclusion level, there was a slight decline in intake by 0.5%. CP intake, on the other hand, increased significantly as a result of Euglena inclusion in the diet by 17.9% from non-supplemented diet to 15% Euglena-supplemented diet. Euglena inclusion in the diet had a significant influence on GE intake, the intake significantly increased by 10.4% from the control diet to the one that contained 15 % Euglena. Intake of the rest of the nutrients tended to increase significantly with increment of Euglena inclusion in the diet up to 10% Euglena-included diet but all recorded a slight decline when the concentration of Euglena in the diet increased by a further 5%.

This study's results were concordant with that of Khandaker *et al.,* (2011) which showed a significant increment in CP intake. However, in their study, there was an insignificant change in DM intake which was explained by the fact that in their study, the diet was fed to cows ad-libitum unlike in the present study which was at maintenance level. The present study's results on DM intake differed with that of Michelle *et al.,* (2012) which showed a decline in DM intake but they accentuated that the fatty acid content of their experimental diet was too high in comparison to the present study. The reduction of DM intake in their study was attributed to the toxicity of high fatty acids content to the rumen microbes. To corroborate the present study, the fatty acid content of Euglena was considerably low as compared to the aforementioned study and probably non-toxic to the rumen microbes and hence significant influence on DM intake. In the present study, as Euglena's fatty acid increased above 10% inclusion level, there was a slight decline in all nutrients intake except on CP and GE intake, this effect could be as a result of mild toxicity to the rumen microbes.The present study's results were congruent with study by Panjaitan *et al.,* (2014). They found that algae supplementation resulted in an increment of feed intake which was coupled with reduced digesta retention time. The study emphatically indicated that DM intake's response to algal supplementation peaked at a certain level of supplementation, which was also in agreement with our study. The present study's CP intake response ensuing Euglena inclusion in the experimental diet showed similarities with that of Khandaker *et al.,* (2011) & Suksombat *et al.,* (2014). In both studies, CP intake increased significantly as a result of increased oil and protein inclusion in the diet. Additionally, supplementing the experimental diets with supplements containing high protein and lipids has been shown to result in a higher CP intake and significant influence on DM intake (Ponnampalam *et al.,* 2005). The present study used Euglena as a supplement which contains high protein and lipid and the results on the aforementioned two nutrients depicted similar responses toward such supplementation. The increased CP intake spurs the growth of rumen microbes which in turn results in a higher intake and digestibility of other nutrients as reported by Degu *et al.,* (2009). The present study believes that the significant influence on other nutrients intake was prompted by the increased rumen microbial population especially bacteria which in turn facilitated digestion. However, at a higher

Euglena supplementation level, there is an accretion of toxicity from the increased oil content which could have occasioned a slight decline in intakes of other nutrients in T4 (15% inclusion level).

5.1.1.2 Nutrients digestibility

Euglena supplementation did not have great effects on some nutrients digestibility neither on the amount digested. The amount of CP digested increased steadily as Euglena inclusion in the experimental diets increased. That is, the CP digested increased from about 66 g/day in T1, the non-supplemented one, to 83 g/day in T4 representing a 25.8% increment. The amount of DM, OM and DE responded significantly to Euglena supplementation. Euglena supplementation did not have a significant influence on the amount of NDF and ADF digested. CP digestibility increased moderately by 4.3% ensuing Euglena inclusion in the experimental diets while GE's digestibility depicted significant response. Other nutrients' digestibility (DM, OM, NDF and ADF) responded insignificantly to Euglena supplementation.

The present study's CP digestibility was in agreement with that of Yang *et al.,* (2007), where oil inclusion in the Holstein-Friesian diet resulted in an increment of ruminal digestibility by 11%. In their study, they found that there were no supplementation effects on DM, OM, and ADF which was also congruent with present study's findings. The above results have also been documented by Kozelov *et al.,* (2001), they found that sheep fed with an oil-supplemented diet had a higher protein in circulation including in the rumen fluid but there were no significant effects on nutrients digestibility. Additionally, this study's results on CP digestibility were consistent with Shinkai *et al.,* (2012) experiment's results which showed a significant increase on CP digestibility ensuing cashew nut oil inclusion into the experimental diet. However, the present study's results on DM and GE digestibilities differed with that of Shinkai *et al.,* (2012), where both nutrients digestibilities declined ensuing cashew nut oil inclusion in the diet, albeit they had also incorporated silicon in the diet in the first trial which they suggested could have reduced some vital rumen bacteria; in the second trial, there was no significant difference among treatments. Mehra *et al.,*(2006) found that supplementary protein resulted in a significant increase in energy intake and digestibility. The present study had an almost the same trend where supplementary protein from Euglena resulted in a significant increase in GE intake and significant energy digestibility. The present study's results further showed consistency with that of El-Gandy *et al.,* (2015), where algae supplementation at different levels resulted in an increment of CP digestibility. Studies by Kawashima *et al.,* (2007) and Bohnert *et al.,* (2007) found that as proteinous supplementation level increased in the experimental diet, fiber digestibility was significantly influenced. However, the present study's findings on the insignificant response of NDF digestibility as protein level in the diet increased are in disagreement with their results. High protein in the diet may trigger alteration of the bacterial population in the rumen which in turn may significantly affect the amount of nutrients digested such as DM and OM (El-Gandy *et al.,* (2015) and Khandaker *et al.,* (2011)). This study strongly believes that the increased protein content of the diet ensuing Euglena supplementation is a plausible cause of this finding where an increment of CP intake concomitantly led to a significant CP digestibility increment coupled with a significant effect in GE digestibility.

5.1.1.3 Effects of Euglena supplementation on protein utilization effectiveness

Euglena supplementation had pronounced effects on protein utilization efficiency. As aforementioned, CP intake increased markedly as Euglena inclusion level in the experimental diet increased. The amount of protein digested and digestibility concomitantly increased with the increment of Euglena concentration in the diet. Urinary protein loss tended to significantly increase but with 15% inclusion level, the loss declined appreciably. The same trend was observed in total proteinous loss (urinary plus fecal CP loss), where the nitrogen loss tended to significantly increase till 15% inclusion level halted the trend. However, Euglena supplementation did not have any effect on fecal CP loss. The amount of protein retained increased steadily as Euglena percentage in the diet increased. Intriguingly, at 15% Euglena inclusion level in the diet, the amount of protein retained perceptibly burgeoned.

Nitrogen (N) utilization efficiency by ruminants is a crucial topic in an environmental perspective. It has been documented that urinary N is more potent as a pollutant than fecal N since it is rapidly degraded by ureases contributing to superfluous N load in the environment (Tomlinson *et al.,* 1996). Therefore, researchers should indefatigably try to concoct a strategy to minimize urinary N loss. This study's findings on a significant effect on urinary N losses and insignificant fecal N losses were congruent with that of Castillo *et al.,* (2001). They found that increase in CP intake and degradability tended to significantly increase urinary N excretion while not affecting fecal N loss. However, N retention in their study was not affected unlike in this study where it increased significantly. This study's results were partly in agreement with that of Bohnert *et al.,* (2007) and Cole *et al.,*(2005), where they found that increased CP in the experimental diets resulted in a significant increment of both urinary and fecal N losses. The present study's results on N excretion contrasted partially with that of McGuire *et al.,* (2013). They found that supplemental N and supplementation frequency did not have any significant effect on both fecal and urinary N excretion. However, both studies had similarities regarding significant N retention increment with the increase of protein supplementation.

5.1.1.4 Effects of Euglena supplementation on energy metabolism

Euglena supplementation had a substantial effect on energy metabolism; various parameters of energy metabolism responded differently. Notably, GE increased significantly as the level of Euglena concentration in the experimental diet increased while DE intake responded significantly as the level of Euglena supplementation increased. Fecal energy loss tended to increase significantly but in treatment four, there was a slight decline. ME intake, urinary, and total energy loss remained unaffected by Euglena supplementation. Total and fecal energy loss as a proportion of GE intake responded significantly to Euglena inclusion in the diet.

The present study had similarities and disparities with other studies, for instance, a study delving into the effect of protein supplementation on energy balance in buffaloes by Mehra *et al.,* (2006) found that ME intake increased while fecal energy loss decreased ensuing protein supplementation. This study was somewhat consistent with Machmüller *et al.,* (2000) where various oils supplementation had manifold effects on energy intake, utilization, and loss.

Contrary to the present study, where GE significantly increased and DE intake was significantly affected by the increased level of oil inclusion in the diet, GE and DE remained unaffected by oil inclusion in their study. However, the studies had similar results on energy loss trend and responses. In both, fecal energy loss responded significantly to oil inclusion while the urinary fecal loss was not significantly affected. Different oils have been shown to affect energy utilization in a different way, but most of them tend to have a similar trend. A study by Obitsu *et al.,* (1995) found that energy intake and utilization were differently influenced by the type of oil infused into abomasum. In their study, GE tended to decrease when some oils were infused, they associated the effect with decreased DM intake. However, in the present study, DM intake tended to increase up to when 15 % Euglena was included in the diet and DM intake slightly declined. Hence, this might be the reason why GE in the present study was only congruent with the oil that did not negatively affect DM intake. Further, the present study was in agreement with the latter cited work on DE, where it was significantly affected in both studies. In stark contrast, the present study's ME was unaffected by oil inclusion in the diet while in their study it was significantly affected by oil abomasal infusion.

5.1.2 Effects of Euglena supplementation on rumen fermentation parameters

5.1.2.1 pH

Euglena supplementation did not have an overall rumen pH effect, pH was within the normal range and averaged out to 6.628±0.104. Euglena inclusion in the experimental diet had an intriguing ruminal pH values pattern in relation to the post-feeding time and inclusion level. There was an intermittent significant influence on pH values as time elapsed post morning feeding, though the pH values were within the normal range. At 24 hours, there was marked ruminal pH increment that averaged out at 7.165±0.075. Throughout the study, pH averaged out at 6.628±0.104 and there was no any recording of pH 6, except at the 24th hour where pH upsurged. In all the other sampling time it was withing the region of 6.16 to 6.87. This pH range has been reported to be adequate to create an auspicious ruminal environment for fiber digestion and support cellulolytic bacteria (Mould *et al.,* 1983).

Studies by Michelle *et al.,* (2012), Vakili *et al.,* (2013) and Chaves *et al.,* (2008) found that oil inclusion in the experimental diet did not have any significant impact on the overall rumen fluid pH. Proteinous supplementation into the experimental diet has been shown not to significantly affect the overall rumen fluid pH (Canesin *et al.,* (2014); Joomjantha and wanapat, (2008)). Canesin *et al.,* (2014) accentuated that there was an intermittent significant influence on rumen pH before and ensuing supplementation before rumen fluid pH eventually stabilized; this is somewhat similar to what this study found. The aforecited work corroborates the present study's findings on rumen pH. However, studies have reported that there could be a significant influence on rumen pH depending on the oil-time interaction. It has been documented that at a higher oil-dose inclusion in the experimental diet, there has been a significant influence on rumen pH and especially at the 24th hour post morning feeding (Castillejos *et al.,* (2006) and Vakili *et al.,* (2013)). However, this study's result partially differs with the aforecited studies on the basis that even at somewhat lower lipid level, pH value burgeoned at the 24th hour and there was no

significant difference among the rumen pH values at the 24th hour. Additionally, the study did not evaluate VFA on a two-hour interval basis but rather it assessed the overall and specific VFA at the 24th-hour rumen fluid collection. It has been reported that a significant fluctuation and variation of VFA concentration may occur among the sampling times and could trigger a change of rumen fluid pH (Canesin *et al.,* 2014). This study is adamant that the intermittent significant difference in pH among the treatments as time elapsed post-feeding was occasioned by the fluctuation of VFA as previously described. The aforementioned studies had intimated that the direct negative correlation between VFA and rumen fluid pH usually exists and such proposition had also been documented by Allen, (1997). Hence, we could not adequately evaluate whether the observed upsurge in pH at 24th hour had been spurred by VFA quantities in the rumen or had a direct correlation as reported.

5.1.2.2 VFA

TVFA in the rumen remained unaffected by Euglena inclusion in the diet. Euglena supplementation to the experimental diet did not exert any significant influence on VFA's concentration in the rumen fluid except the case of valeric which was significantly affected by the supplementation. The ratio of propionic to acetic acid was also not significantly affected by Euglena inclusion in the experimental diet.

The findings of this study on TVFA were in agreement with manifold studies (Patra, (2013); Vakili *et al.,* (2013); Chaves *et al.,* (2008) and Thao *et al.,* (2014)). In their studies, they found that inclusion of essential oil in the experimental diet did not have a significant impact on TVFA. However, in some, there was a significant influence on specific VFA ensuing essential oil supplementation. Interestingly, essential oil inclusion in the latter two studies did not influence the overall rumen fluid pH which was congruent with the present study's findings and as it was reported by Allen, (1997), there is a correlation between the two rumen fermentation parameters. Additionally, to underpin this study's findings on TVFA, studies investigating the effects of proteinaceous supplement in the ruminants' diet have reported an insignificant change on TVFA concentration (Joomjantha and Wanapat, (2008); Canesin *et al.,* (2014)). They postulated that proteinaceous supplements do not exert any significant influence on rumen fermentation's patterns. Intriguingly, the aforecited studies found that the overall rumen fluid pH was not significantly affected by nitrogenous supplements inclusion in the diet, which corroborates this study's finding on pH. However, a study by Bento *et al.,* (2016) about the effects of protein supplementation on ruminal parameters was in stark contrast with this study's findings, where they reported a significant increment of TVFA concentration ensuing proteinous supplementation. Their study was in agreement with the present study since pH was unaffected by the proteinaceous supplementation in their study.

A study by Aemiro *et al.,* (2016) which delved into the effects of Euglena supplemented diet on ruminal fermentation parameters found that TVFA remained unaffected by even up to 400 g/kg DM of the diet, albeit there was a significant reduction of TVFA when the substrate was entirely replaced with Euglena. In their study, specific VFA were influenced significantly by Euglena supplementation including valeric which was also influenced in this study. However, unlike in the present study where the ratio of propionic to acetic acid was unaffected, in the aforecited

study, it was significantly affected by Euglena inclusion in the experimental substrate. Vakili *et al.,* (2013) reported that valerate concentration was not significantly different among the treatments even though they had found that TVFA did not significantly change ensuing oil addition in the experimental diets.

5.1.2.3 NH_3-N concentration

Overall NH_3-N concentration increased significantly by 58.4% from 41.8 mg/L in T1 to 66.2 Mg/L in T4. On the two-hour base, there was a significant influence on overall NH_3-N concentration in rumen fluid. At 0 hour, NH_3-N concentration had the same trend as the one depicted by the overall NH_3-N concentration while in the other sampling hours, there was no any distinct trend, albeit there was a significant difference among the treatments.

Khandaker *et al.,* (2011) found that NH_3-N concentration was increasing steadily and especially few hours post-feeding. They indicated that the apotheosis of the increment was at the 3rd-hour post-feeding and a gradual decline was observed at around 7th hour. Although this study did not delve into the trend of NH_3-N concentration, numerically, it seemed to be congruent with their study. Further, they reported that NH_3-N concentration was increasing significantly as the level of supplementation increased, this was in agreement with the present study. However, this study's results on NH_3-N concentration was in stark contrast with that of Sallam *et al.,* (2011), where they reported plummeting of NH_3-N concentration ensuing Essential Oils (EO) supplementation. They conjectured that the drastic decline in NH_3-N concentration could have been spurred by EO of *Mentha microphylla* which could reduce amino acid deamination. Reduced amino acid deamination increases ruminal escape of protein hence improving efficiency of N utilization in the rumen.

Proteinaceous supplementation has been shown to significantly affect NH_3-N concentration. Period and Leng, (1990) and Kim *et al.,* (2009) reported an increment of NH_3-N concentration as the level of supplementation increased. In the latter cited work, they correlated the increment of urinary N excretion with the increase of NH_3-N concentration in the rumen. To underpin their postulation, they stated that the increased urinary N excretion is as a result of an inadequate capture of ruminal NH_3-N for microbial protein synthesis ensuing increased N intake. This study strongly believes that the same notion is plausible to explicate N metabolism and NH_3-N concentration in the rumen as the level of Euglena supplementation increased. In addition, Bohnert *et al.,* (2002) also pinpointed an increase of NH_3-N concentration in rumen triggered by an increased level of proteinous supplementation.

Previous studies investigating the effects of microalgae on rumen fermentation parameters reported a significant influence of microalgal inclusion in the experimental diet on NH_3-N concentration. Panjaitan *et al.,* (2014) reported an increment of NH_3-N concentration ensuing spirulina algae supplementation. The increment was ascribed to a high level of algae supplementation which implied high protein content in the experimental diet. Aemiro *et al,* (2016) also reported an upsurge of NH_3-N concentration when the level of Euglena supplementation increased. They associated the surge of ruminal NH_3-N concentration with the increased CP in the experimental diet as a result of Euglena inclusion.

A study by Sallam *et al.,* (2011) hypothesized that NH_3-N concentration in the rumen may decrease or increase depending on the amount of degradable proteins available in the rumen. The gradual decline in the overall NH_3-N concentration in T4 and in all two-hour sampling periods may be associated with presumably improved N utilization in the rumen by bacteria. The aforementioned inference can be underpinned by the facts that CP retained increased significantly ($p<0.05$) in T4 while urinary N lost in T4 was significantly affected ($p<0.001$) and numerically reduced. That N utilization efficiency may have been spurred by the significant decline in protozoa which are said to be the predators of some bacteria that are essential for NH_3-N conversion into microbial proteins. Additionally, protozoas are said to be the producers of a substantial quantity of rumen ammonia (Sallam *et al.,* 2011) and hence, with their numbers in rumen decreasing, it directly reduces ammonia production and relieves bacteria from predation which in turn bolsters NH_3-N conversion into microbial proteins (Abubakr *et al.,* 2013). Hristov *et al.,* (1999) attributed the lower ruminal NH_3-N concentration to reduced protozoa numbers in the rumen. The trend of higher NH_3-N concentration few hours post-feeding and gradual decline as time elapsed could be due to the immediate feeding effects in the rumen as it was previously intimated by Islam *et al.,* (2000). Throughout the experiment, the concentration of NH_3-N remained within generally required concentrations for efficient rumen fermentation of fibrous substrates, which is about 50 mg/liter.

5.1.2.4 Rumen Protozoa

Euglena supplementation markedly affected protozoa population in the rumen, the population perceptibly plummeted following inclusion of Euglena in the experimental diet. Protozoa numbers in the rumen slumped from about 3.016 cells per milliliter(ml) in T1 to about 1.075 cells per ml in T4, which represented a 64.4% decline.

Manifold studies delving into the effects of oil inclusion in the diet on rumen protozoa population have reported varied impacts. Most have reported a decline in rumen ciliate protozoa population ensuing oil supplementation while others have recorded an unchanged protozoal population. Thao *et al.,* (2014) and Abubakr *et al.,* (2013) reported a slump in rumen ciliate protozoa population in their *in vivo* studies. In fact, the latter cited work reported that protozoa numbers plummeted and eventually they were eliminated.

In vitro studies have also reported somewhat similar results to those reported by *in vivo* studies. Sallam *et al.,* (2011) and Jal *et al.,* (2002) reported the same ruminal protozoas decline following an inclusion of oils in the experimental diet. The formerly cited literature postulated that oil has pronounced effects on rumen fermentation in general and particularly affects protozoas. An *in vitro* study by Joaquin *et al.,* (2015) found that protozoal population was decreasing after every transfer from one batch to another, they even extrapolated the effect and surmised that protozoas could not have survived beyond fifth batch transfer. They ascribed that trend to the toxicity of oils to rumen protozoas.

Microalgae, which are considered as an alternative lipid and/or protein source for ruminant's diets (Tsiplakou *et al.,* 2016), have been reported to have a significant effect on ciliate protozoa

population. Boeckaert *et al.,* (2007) reported a decrease in ciliate community ensuing algae inclusion in the dairy cows' diet. In addition, they accentuated the marked change in ciliates diversity following algae supplementation. The aforecited literature is in agreement with the present study, where the protozoal population decreased markedly ensuing Euglena supplementation. The latter cited work inferred that algae have an acute effect on rumen ciliate community and present study corroborated that.

Specific algae supplementation to the ruminant diets has been reported to have similar results on rumen ciliates community. Aemiro *et al.,* (2016) documented that protozoa population decreased by up to 44.8% ensuing Euglena addition into the experimental substrate in their *in vitro* study. The marked decrease in protozoa population was ascribed to a significant concentration of unsaturated fatty acids. Boeckaert *et al.,* (2007) conjectured that ciliates in association with bacteria might be playing a critical role in biohydrogenation of unsaturated fatty acids. They further accentuated that accumulation of hydrogenation intermediates could trigger changes in rumen ciliates, hence underpinning the cause of protozoa decrease following algae supplementation to the experimental diet.

5.2 *In vitro* study

5.2.1 Effects of Euglena supplementation on CH_4 emission

CH_4 emission declined numerically in a dose-dependent manner by up to 35.4% following 20% Euglena inclusion level in the experimental diet. Though the decline was not statistically significant, it was numerically perceptible. Hence, the study was adamant that there was an existence of a positive interaction among the experimental substrates, ruminal microbes and the supplement that resulted in that appreciable CH_4 emission decline. The level of CH_4 emission decline was within the region reported by other studies that utilized microalgae to mitigate CH_4 emission. The studies by Maia *et al.,*(2016), Dubois *et al.,* (2013), and Kinley and Fredeen, (2015), which delved into utilization of various types of microalgae to abate CH_4 emission, found varied results. The former cited literature reported a reduction by 35.8% by some seaweed while the latter reported an average reduction of CH_4 emitted by 16%. The latter cited work accentuated that some seaweeds triggered CH_4 reduction, albeit not statistically significant. The reduction in CH_4 emitted ensuing seaweed supplementation has been associated with the fact that they have high protein, oil content, and bioactive compounds which have various antimicrobial activities (Kumar *et al.,* 2008). Dubois *et al*., (2013) intimated that inclusion of protein in the experimental diet as a replacement of fiber reduces total gas production including CH_4 while as lipid replacement, CH_4 would increase. In the present study, Euglena supplementation resulted in a reduction of crude fiber content by 9.2% and an increment of lipid by up to 85.7% ensuing 20% Euglena inclusion in the experimental diet that lead to increment of CP by 21.2%. Cone and Gelder,(1999) illuminated how fermentation of proteins leads to a lower gas production in comparison with carbohydrates or fiber. The Increased CP content in the present study's experimental diet is highly believed to have contributed to reduction of CH_4 emission. This is because dietary CP and NDF content of the diet have been shown to affect the amount of OM fermented and hence affecting CH_4 emission. The Increased crude fiber content suppresses

microbial activity, affects the fermentability of the diet, and in return results in high CH_4 production per unit of OM digested. On the other hand, if the CP content of the experimental diet is high and soluble, it improves microbial activity, fermentability and hence reducing the amount of CH_4 produced per unit of OM digested (Meale *et al*., 2012).

Manifold studies delving into utilization of various essential oils to abate CH_4 emission have been conducted and published. They have documented varied effects and magnitude of CH_4 reduction. Benchaar and Greathead, (2011) explicated how various volatile lipophilic components of essential oil exert effect on microbes especially methanogens. They illustrated the mechanism of action exerted by various oil's lipophilic component. However, they accentuated that the sensitivity of microbes to oils varies and that is why there could be varied response ensuing oil supplementation. A study by Patra and Zhongtang, (2012), testing the potencies of five different essential oils in three different doses, had validating results to the aforecited literature. They found that CH_4 production and emission was significantly and differently reduced by different oils with increasing doses. They attributed the reduction of CH_4 emission to antimethanogenic property possessed by essential oils; the potency varies and the sensitivity of methanogens to the oil also varies markedly. A review by Grainger and Beauchemin, (2011) gave an appraisal on how various fat and levels of inclusion in the experimental diet affect ruminal fermentation and eventually reduce CH_4. They suggested that microalgae would reduce CH_4 due to high content of omega-3 and 6 fatty acids which have been shown to reduce CH_4 production. The Euglena supplement used by this study had a substantial content of omega-3 and 6 as shown in the Table 4.1.5 above which probably prompted a reduction of CH_4 emission.

The high oil content of Euglena is also highly believed to contribute to reduction of CH_4 emitted; supplementation of Euglena by up to 20% resulted in an increment of EE in the experimental diet. Dietary lipids effects on CH_4 have been shown to reduce CH_4 and the magnitude of the effect hinges on the source of the oil, inclusion level, fatty acids profile and the composition of the diet (Beauchemin *et al.,* 2009). There have been several postulations about the mechanism of CH_4 reduction by dietary lipids. Firstly, dietary lipids are believed to have anti-microbial activity. Various components of dietary oil have a broad spectrum of effects on methanogens (Helander *et al.,* 1998), with an eventual result being the reduction of CH_4 production. Fatty acid profile such as polyunsaturated fatty acids negatively act against various microbes such as lactate producers, while favoring bacteria that produce propionate in the rumen (Frater, 2014). This results in an alternative hydrogen sink other than that of CH_4 production. Methanogens grow by oxidizing H_2 and reducing CO_2 to CH_4; so in case an alternative H_2 sink is created, CH_4 production and emission eventually reduce. In addition, polyunsaturated fatty acids are said to be having toxic effect on protozoa and cellulolytic bacteria (Frater, 2014). The decline in protozoa contributes indirectly to the reduction of CH_4 production; since about 25% of the rumen methanogenesis is triggered by methanogenic bacteria that are closely associated with protozoa (Newbold *et al.,* 1995). The methanogenic archaea that are closely associated with protozoa are mostly on the rumen ciliates' external surface and have endosymbiosis with protozoa. They ensnare H_2 produced by protozoa to produce CH_4 (Tan *et al.,* 2011). Any feed supplement that have antiprotozoal effect will concomitantly reduce such endosymbiotic methanogens, and therefore resulting in a low CH_4 production and emission.

Secondly, biohydrogenation of polyunsaturated fatty acids that involves rumen bacteria offers an alternative utilization of H_2 produced as an end-product of rumen fermentation and by protozoa. This reduces the H_2 gas in the H_2 sink and hence reducing the amount of H_2 that is at methanogenic archaea's disposal. Hobson and Stewart, (2012) elucidated that even though biohydrogenation does not compete effectively with methanogens for H_2, it utilizes ruminal H_2 substantially. Deducing from their literature, increment of polyunsaturated fatty acids in the experimental diet will eventually lead to substantial utilization of H_2 hence reducing the abundance of H_2 available for methanogenesis. This study utilized experimental diet's supplement that had a substantial amount of unsaturated fatty acids; based on above scientific evidence, the study is adamant that the unsaturation of fatty acids had a hand in reduction of CH_4. A study by Aemiro *et al*., (2016) highly ascribed the CH_4 reduction in their study to the unsaturated fatty acids in the experimental diet. Their study utilized Euglena as a supplement which had a considerable proportion of unsaturated fatty acids. They attributed the CH_4 reduction in their study to the presence of high amount of fatty acids especially unsaturated fatty acids. Their study recorded 9-48 percent reduction of CH_4 depending on the level of Euglena supplementation. This is a corroboration that the current study's result were within the possible reduction that Euglena supplementation can contribute.

5.2.2 Effects of Euglena supplementation on simulated rumen fermentation parameters

5.2.2.1 VFA

VFA form a substantial metabolizable energy source for ruminants following absorption across rumen wall (Yang *et al.,* 1970). They form vital end-products of rumen fermentation and hence any feed supplement that is intended to exert influence on the rumen fermentation must not adversely affect VFA production. This study's supplement did not significantly affect TVFA; in fact, TVFA increased numerically after 20% inclusion level. The fermentation process favored acetic production over propionic. Isobutyric and isovaleric numerically increased, an indication that the fermentation of protein greatly influenced the nature of fermentation since those VFA are the end-products of protein fermentation (Hungate, 1966).

Oil inclusion into the experimental diet with an intent to lower CH_4 has been reported to have a wide range of effects. Published literature about the effect of essential oil on VFA by Hundal *et al*., (2016), and Benchaar and Greathead, (2011) documented that oil inclusion in the experimental diet significantly depressed TVFA, individual VFA, and the ratio of acetate to propionic (A:P). The deleterious effect of oil supplementation on fermentation parameters exacerbated with an increasing dose of inclusion. This was somewhat differing with the current study's result where TVFA and individual VFA were unaffected statistically. Even at a higher dose of inclusion, the effect was not statistically significant. That implied that Euglena oil reduced CH_4 numerically without compromising fermentation process markedly. A study by Tekeli *et al.,* (2015) which determined the efficacy of essential oil on VFA illustrated that oil inclusion into the diet at lower dosage did not affect TVFA and individual VFA, but at a higher dosage there was a significant reduction of TVFA suggesting that though the oil inclusion may

abate CH_4 emission, the level of inclusion must be considered methodically. The decline of VFA is attributed to a low microbial activity in the rumen that is occasioned by the toxicity exerted by the oil and/or to reduced digestibility of the digesta.

Protein fermentation in the ruminal condition is said to influence TVFA and individual VFA especially isobutyric and isovaleric according to Hungate, (1966). The current study's findings are in agreement with Maccarana *et al.,* (2016). In their study, the increased proportions of diet CP did not adversely affect TVFA or individual VFA. However, they underscored that isobutyric and isovaleric increased as a result of diet CP increment which was also reported by Boeckaert *et al.,* (2009). The current study found that the aforementioned individual VFA tended to increase numerically even if the contrasts did not show statistical significance of the increment. The present study believes that the response by the two VFA was as a result of CP increment in the experimental diet. Further, the present study's findings were in strong agreement with the study by Norrapoke *et al.,* (2012) that appraised the effect of protein level on rumen fermentation. They emphatically stated that protein levels did not significantly affect TVFA and individual VFA; that was in agreement with the present study's findings. Additionally, the present study's findings on the range of individual VFA were congruent with their study and Hungate (1966). The current study found that acetic, propionic and butyric acid concentration averaged out at 67.6%, 23.1% and 7.1% of TVFA respectively while the aforecited literatures were in the region of 62-67.8%, 21.2-22% and 11.1- 16 %.

Microalgae alter rumen fermentation and affect TVFA and individual VFA depending on the level of supplementation (Zhu *et al,* 2016). At a lower inclusion dose, there were no pronounced effects on TVFA or specific VFA but when the dose increased, TVFA concentration declined. The response is attributed to an increment of polyunsaturated fatty acids in the experimental diet. Aemiro *et al.,* (2016) documented that there was no significant effect on TVFA and individual VFA ensuing Euglena supplementation at different levels of inclusion. The present study's findings were in agreement with the aforecited literature which validates the current study's findings.

5.2.2.2 NH_3-N

Euglena supplementation to the experimental diet modified the simulated ruminal fermentation; consequently, the supplementation affected the end-products of the fermentation process. One of the significantly affected end-products of fermentation was NH_3-N, which significantly increased ensuing Euglena inclusion in the diet. NH_3-N increased in a dose-dependent manner; the increment was by 67.3% after 20% Euglena inclusion in the diet. NH_3 is formed in the rumen ensuing conversion of peptides and amino acids by ruminal microbes. If there is excessive breakdown of peptides to NH_3, it is construed as nutritional inefficiency, since over-produced NH_3 is lost across the rumen and energy is a prerequisite in that microbial process (Wallace *et al.,* 1999). However, the breakdown of peptides to a certain extent is beneficial since the formation of microbial N hinges on the presence of both NH_3 and peptides in different proportions depending on various conditions (Wallace *et al.,* 1999).

A myriad of studies have reported wide-ranging findings on NH_3-N following inclusion of oil in the experimental diet. Studies by Abubakr *et al.,* (2013), Benchaar *et al.,* (2007), and Gunal *et al.,* (2014) utilizing various essential oils in different conditions, and in different levels of inclusion found different responses to the oil inclusion in the diet. Benchaar *et al.,* (2007) reported a decline, Abubakr *et al.,* (2013) documented an unaffected NH_3-N concentration while the latter literature reported an increment of NH_3-N ensuing essential oil inclusion in the diet. The decline was attributed to a lower deaminase activity, the lack of effect was slightly associated with the level of supplementation and the experimental procedure whereby rumen microbes can adapt to oil in the diet while the increment was ascribed to the increased deamination of amino acids by Hyper-ammonia Producing bacteria (HAP) which was said to be less sensitive and possessing super-deamination activity. The current study's findings on NH_3-N suggest that the oil in the experimental diet did not adversely affect HAP which could be due the level of supplementation or HAP were not very sensitive to the level of EE in the experimental diet.

Microalgae supplementation has been reported to significantly affect NH_3-N concentration in the rumen, which has been construed to be a resultant effect of increased protein in the diet. Panjaitan *et al.,* (2014) reported an increment of NH_3-N following microalgae inclusion in the animal diet. The increment was attributed to the increase of protein especially Rumen Degradable Protein (RDP). A study by Maia *et al.,* (2016) utilizing seaweeds to manipulate rumen fermentation reported similar results of NH_3-N increment, which they associated with a higher CP in the supplement than in other experimental substrates. Kim *et al.,* (2009) confirmed that increased CP in the experimental diet results in NH_3-N increment. The current study's CP in the experimental diet increased by 21.2% following 20% Euglena inclusion in the diet. A study by Aemiro *et al.,* (2016) investigating the effects of *Euglena gracilis* on CH_4 and rumen fermentation parameters reported an increment of NH_3-N in the fermentation medium as the level of supplementation increased. They ascribed the escalation of NH_3-N to an increase of CP in the experimental diet as Euglena content in the diet increased. The improvement of CP in the current study's experimental diet is strongly believed to have spurred an increment of NH_3-N concentration in the fermentation medium.

5.2.2.3 Protozoa

Rumen protozoa, predominantly ciliates, seem to contribute to rumen fermentation in an unclear role. A raft of published literature have tried to elucidate the roles of rumen protozoa, however, indistinctly. The striking role of the ciliates has been reported to be predation of the bacteria to offset overgrowth. Protozoas make a substantial portion of rumen biomass somewhat similar to the bacteria despite their difference in numbers. Protozoa are highly associated with at least 25 percent of rumen methanogens (Newbold *et al.,* 1995). There are various postulations that have been documented to underpin close association of protozoas to enteric methanogenesis. The suppositions include H_2 producing ability in hydrogenosomes, epi-and endosymbiotic methanogens hosting and protection ability of protozoas, and interspecies H_2 transfers between protozoas and symbiotic bacteria (Newbold *et al.,* 2015). Now that CH_4 abatement research has gained traction among ruminant nutritionists, research tend to leverage the close association of protozoa and methanogens to reduce CH_4 emission. A meta-analysis by Guyader *et al,* (2014)

established that there is a significant linear relationship between protozoa concentration in the fermentation medium and CH_4 emitted. However, they pointed out the existence of other methanogenesis abatement mechanisms that does not involve protozoa, albeit only 21% of the total 76 experiments reported it.

Protozoa population in the current study's fermentation medium decreased numerically as Euglena inclusion in the diet increased, albeit the contrasts revealed that the decline was not statistically significant. The study's substrate EE increased by up to 85.7% following 20% Euglena inclusion in the experimental diet. The increased EE content of the diet is believed to have contributed to the reduction of protozoa population in the fermentation medium. Studies by Abubakr *et al*., (2013) and Patra and Zhongtang, (2012) evaluating the effects of essential oils on fermentation parameters reported a decline of protozoa population. The reduction magnitude hinged on the type of oil, the level of supplementation, and the sensitivity of protozoa to dietary fat. An adequate dietary lipid in the diet is believed to be toxic to rumen protozoa, though the potency differs. The mechanism of dietary lipids toxicity to protozoa is basically based on their inability to absorb and transform lipids properly resulting in the swelling of protozoa cells and subsequent rapture. A meta-analysis by Guyader *et al.,* (2014) documented that 31% of the 76 experiments that utilized lipids to manipulate rumen fermentation had a concomitant CH_4 emission and protozoa reduction. This confirms that dietary lipids possess toxic effects towards protozoas and have different potencies.

Microalgae, which are reported to have higher EE than ordinally substrates, have been reported to result in protozoa reduction following their utilization as supplements. Boeckaert *et al.,* (2007) found that microalgae supplementation resulted in protozoa reduction and even their diversity was affected. They associated the reduction with accumulation of biohydrogenation intermediates. Aemiro *et al.,* (2016) reported a decline in protozoa population by 14.8-44.8% ensuing Euglena inclusion. The present study's results showed that protozoa declined by up to 21.4% ensuing 20% Euglena inclusion level, which was within the range reported by the latter cited literature.

5.2.2.4 pH

The fermentation medium's pH in the current study was unaffected by Euglena supplementation; the pH was in the region of 6.98±0.01, which was regarded as an auspicious ruminal pH for fiber digestion and cellulolytic bacteria activities by Mould *et al.,* (1983). A number of studies have reported unaffected pH ensuing oil inclusion into the experimental diet. Studies by Thao *et al.,* (2014), Yang *et al.,* (2007), and Chaves *et al.,* (2008) delving into the effects of oil inclusion in the experimental diet reported unaffected ruminal pH. The current study's experimental diet lipid content did not affect the fermentation medium pH which is congruent with the aforecited studies.

Proteinous supplements such as microalgae do not affect ruminal pH according to a raft of studies. Boeckaert *et al.,* (2009), Zhu *et al.,* (2016), and Maia *et al.,* (2016) found that microalgae supplementation did not affect ruminal pH. Further, their study findings were corroborated by Kinley and Fredeen, (2015) who documented unaffected ruminal pH ensuing seaweeds supplementation to the diet. These consistent findings show that increased CP in the

experimental diet ensuing microalgal supplementation does not affect ruminal pH unlike when more soluble carbohydrates are fermented which is beneficial to the rumen fermentation, general animal health, and productivity. Joomjantha and Wanapat, (2008) evaluating the effect of protein-rich feed resources on rumen fermentation reported an unaffected ruminal pH which undoubtedly proves that CP increment in the experimental diet is unlikely to significantly affect ruminal pH. The present study's findings on unaffected fermentation medium pH validates the aforecited literatures.

5.2.2.5 ORP

Rumen or fermentation medium pH and ORP are the two main physicochemical parameters that rumen microbial ecosystem principally depends on (Marden *et al.,* 2005). The two fermentation parameters largely reveal how the microbial fermentation dynamics are. ORP, wich is construed as an indicator of strong reducing power and absence of oxygen, must be very low (Richter *et al.,* 2010). In the present study, ORP reflected the equilibrium between the fermentation medium and the gas mixture in the headspace of the fermentation jars. The present study's ORP values were unaffected statistically but numerically tended to decline as the level of supplementation increased. The unaffected ORP implied that the fermentation jars were maintained auspiciously to support optimal microbial activity without intrusion of environmental gasses into fermentation jar. The present study's ORP values were somewhat within the ranges reported by Richter *et al.,* (2010) and Aemiro *et al.,* (2016), albeit they were not affected significantly as the latter literature reported.

5.2.3 Effects of Euglena supplementation on digestibility of nutrients.

Digestibility of feed signifies the relation between the substrates' nutrient content and the energy that are available to ruminants for absorption. That is the difference between the substrates' nutrient content and residue nutrients content after fermentation expressed as a percentage of substrates nutrients content. Hence, digestibility forms a valuable factor of nutritive value of any feed substrate since it shows how ruminants ingesting that feed substrate would perform (forejtová *et al.,* 2005). The present study delved into the effect of Euglena supplementation on DM and OM digestibility. IVDMD and IVOMD improved as Euglena in the experimental diet increased.

Lipid inclusion has been shown to affect nutrients digestibility depending on the type of oil, the level of supplementation, and the sensitivity of ruminal microbes. Studies by Meyer *et al.,* (2009) and Yang *et al.,* (2007) evaluating the effects of various essential oils in an *in vivo* study reported unaffected digestibilities of both DM and OM.They conjectured that the supplementation dose was not adequate to exert anti-microbial activity in order to significantly affect microbial fermentation of the two nutrients. However, a raft of studies that evaluated effects of oil on *in vitro* nutrients digestibility reported significant nutrients digestibility decline (Vafa *et a*l., (2009), Benchaar *et al.,* (2007), and Kamalak *et al.,* (2011)). They documented that the oil they used

exerted anti-microbial effect that significantly affected the fermentation process. Notably, other fermentation parameters in their study such as pH and VFA were significantly affected unlike in the present study indicating there was adverse effect on the fermentation process. They cited that the potency of the oil's adverse effect on microbial activity was largely influenced by the oil type, the level of inclusion, the degree of saturation, and the chemical composition of the experimental diet. The degree of saturation and the level of oil inclusion have been mainly associated with the decline of nutrients digestibility. Kamalak *et al.,* (2011), Vafa *et al*., (2009), and Aemiro *et al.,* (2016) accentuated that digestibility of nutrients dwindled as the level of lipid in the experimental diet increased, implying that the anti-microbial potency of the oil increased in a dose-dependent manner.

It is, therefore, plausible to surmise that the level of lipid inclusion in the present study's experimental diet was not adequate to adversely affect microbial fermentation of the experimental diet. This could be beneficial in the sense that CH_4 emission could be decreased without affecting microbial fermentation, which is advantageous for ruminant performance.

Proteinous supplements have been reported to either improve DM and/or OM or have no effect at all. Studies by Quang *et al.,* (2015) and Joomjantha and Wanapat, (2008) reported increased and unaffected digestibility coefficients of OM and DM respectively ensuing protein-rich supplement inclusion in the experimental diet. Microalgae are said to be easily digested and utilized by ruminants due to their substantially high DMD coefficients (Anele *et al.,* 2016). Positive effects on nutrient digestibility have been documented following microalgae inclusion in the experimental diet. A study by Machado *et al.,* (2014) reported that IVDMD varied among the various microalgae used, but the difference was not statistically significant. The aforecited study's results are in agreement with the present study's findings since even in the present study IVDMD and IVOMD were significantly improved. The improvement of both IVOMD and IVDMD in the present study is thought to have been occasioned by the improved experimental diet CP. The increased CP in the experimental diet is said to increase microbe population and activity which in turn act on organic matter (El-Gandy *et al*., 2015). Yang *et al.,* (2007) evaluating the effect of various essential oils accentuated that ruminal OM and DM digestibilities were higher than that of control because of increased digestion triggered by improved dietary protein. Fiber content (NDF and ADF) of the experimental diet is also said to influence its digestibility. The increased cell wall content of the diet suppresses microbial activity by limiting availability of carbohydrate to microbes (Meale *et al.,* (2012), and Buxton & Redfearn, (1997)). In the present study, Cell wall content (NDF and ADF) decreased as the supplementation level increased as shown in the Table 4.1.2 above; this is also strongly believed to have prompted improvement of IVOMD and IVDMD. Aemiro *et al.,* (2016) also suggested that the increment of Euglena content from 100 g/kg of DM may contribute to the improvement of OM and DM digestibility simply because of high digestibility of Euglena.

Conclusions and recommendations

Conclusions

After carrying out the two studies, that is *in vivo* and *in vitro*, this study made various meticulous deductions from the results. The inferences that the study deduced from the results include the following;

- Euglena supplementation influenced nutrients intake significantly, specifically CP and GE intake increased as the level of supplementation increased. Other nutrients were influenced by Euglena supplementation differently and the influence primarily hinged on the level of Euglena in the diet. Ruminants that are attuned to Euglena usually ingest Euglena freely; in our study, there was no incidence of Euglena refusal throughout the study.
- Euglena inclusion in the diet improved digestibility of nutrients depending on the level of inclusion in the diet. CP digestibility was perceptibly increased ensuing Euglena supplementation. The effect of Euglena supplementation on CP metabolism, that is intake, the amount digested, and digestibility is believed to be the fulcrum of all the effects that Euglena exert on the fermentation process and affected digestibility of other nutrients. Following supplementation, the cell wall content proportion of the diet reduced appreciably as Euglena content in the diet increased. This is believed to have spurred the improvement of digestibility of the other nutrients like OM and DM.
- Euglena supplementation exerted an intriguing rumen fermentation effect, this is probably the reason why it is touted to be among the best alternative feed supplements. It effectively abated CH_4 emission without exerting a substantial adverse effect on rumen fermentation process. CH_4 emitted reduced markedly but pH and TVFA were not affected, this implied that the supplementation does not jeopardize ruminant productivity but rather improves it while abating enteric CH_4 emitted by ruminants. Rumen protozoa reduced ensuing Euglena supplementation while NH_3-N increased significantly. The increment of NH_3-N in the rumen fluid was because of increased CP in the diet. The

increment of NH_3-N in the rumen fluid is highly associated with the increment of N lost through urine, which is construed as nutritional inefficiency. This indicated that the increased NH_3-N in the rumen could not be entirely ensnared for microbial protein synthesis.

- It is evident from the result that Euglena supplementation resulted in a reduction of CH_4 emitted. Although this study did not conduct an *in vivo* study to evaluate effect of Euglena inclusion in the animal diet on CH_4 emission, it is adamant that the *in vitro* study's results can be extrapolated to an *in vivo* situation since they had marked similarities on the other parameters investigated.
- From all the above compelling effects of Euglena supplementation, it is crystal clear that Euglena can be a beneficial alternative feed supplement from the environmental and animal productivity perspectives.

Recommendations

For any new feed supplement to get acceptability from the stakeholders especially farmers, it should not affect ruminants productivity and profit margin. Those are the characteristics of a supplement that the farmers put on the front burner. Then, whether the supplement is environmentally benign, it comes second to the aforementioned characteristics. With this in mind, the study recommends the following;

- The level of Euglena supplementation should be painstakingly considered; this affects both rumen fermentation and profit margin. As shown by the two studies, the effects of Euglena supplementation highly depended on the levels of supplementation. A high level of supplementation will result in an increment of lipid in the diet to an adequate amount to cause toxicity to cellulolytic bacteria and hence adversely affecting rumen fermentation. It is noteworthy to indicate that a higher CP in the diet resulted in the increment of N lost through urine which is both ineffective nutritionally and perilous to the environment. At a higher level of Euglena supplementation, farmers' profit margin will also narrowed.
- There is a dire need of another research to investigate utilization of defatted Euglena as a supplement on nutrients intake, digestibility, rumen fermentation, and enteric CH_4 emission. In order to reduce the cost of Euglena, improve the profit that the farmers get, and utilize the gargantuan defatted Euglena biomass ensuing oil extraction, there is an urgent need to evaluate whether the defatted Euglena influences the aforementioned parameters. In additional, there is a need for a further research to ascertain precisely which bioactive compound of Euglena and the level of Euglena lipid that is adequate to cause reduction of CH4 emission.

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Appendix

Determination of rumen liquor VFA content.

A) Sample preparation

I. The frozen samples (at -20^0 C) were thawed by dipping them in a container containing tepid water for few hours prior the experiment.
II. 1.5 ml of the rumen liquor was transferred into Eppendorf tubes and centrifuged (18000 revolutions/min for 15 minutes at 4^{0}C).
III. 0.8 ml of supernatant was transferred into another Eppendorf tube.
IV. 0.2 ml of 25% Metaphosphoric acid was added into Eppendorf tubes containing 0.8 ml of the supernatant.
V. The mixture of the two was thoroughly mixed by vortexing and frozen overnight at -20^0 C.
VI. The frozen samples were defrosted the following morning as described by the step I above and then centrifuged (8000 revolutions/min for 15 minutes at 4^{0}C).
VII. Septa were inserted into the vial caps; hard side of septa was placed to be in contact with the glass.
VIII. 0.5 ml of the supernatant was transferred into a 1.5 ml vial glass.
IX. 0.5 ml of 10mM 2-ethylbutyric acid was added into the vial glass containing 0.5 ml of the supernatant. Bubbles were removed before dispensing and dispensing was done carefully to obviate spillages.
X. The samples were then kept in the fridge at 4^{0}C awaiting analysis.

B) Standard preparation

I. Four vial glasses were prepared for standard solutions.
II. Each standard solution contained the following: Acetic (50mM), Propionic (50mM), isobutyric (50mM), Butyric (50mM), Isovaleric (10mM), Valeric (10mM), and Caproic (5mM).
III. To each vial glass, 0.5 ml of standard solution and 0.5 ml 2-ethylbutyric acid were added and mixed by vortexing.
IV. The standards were also kept in the fridge at 4^{0}C until analysis.

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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ミドリムシ(ユーグレナ)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2つの実験を実施した。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、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38.81±4.38kg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、クレイングラス乾草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。

養分摂取量は、ユーグレナの添加によって影響を受けた($P<0.05$)。粗タンパク質(CP)および中性デタージェント繊維(NDF)消化率にユーグレナ添加の影響がみられた($P<0.05$)。CP摂取量、消化率、尿中への排泄量は増大した($P<0.05$)。糞中へのCP排泄量には影響はみられなかった($P>0.05$)。総エネルギー(GE)摂取量は、有意に増加した($P<0.0001$)。ユーグレナの添加によって、ルーメン内アンモニア濃度は影響を受け($P<0.0001$)、プロトゾア数は減少した($P<0.0001$)。ユーグレナの添加は、pHと総VFA濃度に影響を及ぼさなかった($P>0.05$)。

2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。

粗飼料としてケニヤで収穫したキクユグラス6gを基礎飼料とし、濃厚飼料とユーグレナをそれぞれ4gと0ｇ、3ｇと1ｇ、2.5ｇと1.5ｇ、2ｇと2ｇの比率で混合し、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、24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、ユーグレナの添加によって35.4%減少したが、有意なものではなかった(P=0.142)。アンモニア濃度(mg/ml)は、ユーグレナの添加によって増加した(P<0.001)。pHおよび総VFA濃度(mmol/l)は、影響はみられなかった。プロトゾア数は、21.4%減少したが、有意ではなかった(P= 0.161)。人工消化試験による有機物消失率は、ユーグレナの添加によって有意に増加した(P=0.0011)が、乾物消失率は有意な差はみられなかった(P=0.1823)。

以上の結果から、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。また、ユーグレナ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る可能性を持つものといえる。