Methane emission and plasma nutrients metabolism in sheep fed garlic diets

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2017

Methane emission and plasma nutrients metabolism in sheep fed garlic diets

A thesis

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

June, 2017

By

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Declaration

I confirm that the work undertaken and written in the thesis is my own work that it has not been submitted in any previous degree application. All quoted materials are clearly distinguished by citation marks and sources of references are acknowledged.

Acknowledgement

My deepest and warmest thanks and appreciation to my main supervisor, Professor Dr Sano Hiroaki for his endless encouragement, kindness, support and friendship throughout the years of my PhD study. Kita Kazumi as my second supervisor for his continuous support and encouragement to complete my PhD thesis. I would also like to thank my other co-supervisor Horiguchi Kenichi from Yamagata University and Matsuzaki Masatoshi of Hirosaki University who kindly attended various meetings and presentation and provided their valuable support and advice to improve my PhD thesis. I am also grateful to the staff of The United Graduate School of Agricultural Science, Iwate University and International Office at Iwate University for their constant help throughout my stay in Japan. I would also like to thank Ministry of Education, Sports and Culture (MEXT) for financial support through a PhD scholarship scheme. I would also like to thank the Dr. Nakasawa at Regional collaboration research institute for helping me with the analysis of isotopic enrichments.

I would also like to thank Sekiguchi Natsumi, Takamori Kudou.. Kamruzzaman Sajal, Liang Si, Mostafiz Rahman, Paramintra Vinitchaikul, Bi Xue, Matsunaga Bungo, Chitose Watanabe, , Komine Tamami, Ayana Matsuno, Hideyaki Hori for their helps in different experiment and laboratory analysis. I would also like to thank Kim Yohan for helping me learn different laboratory techniques. Also all the Japanese students and friends who have not been written here due to space, you are always there in my heart. You made my stay in Japan warm and memorable and would also like to thank you for all sweet events throughout my stay.

Lastly, I would like to express my gratitude to my beloved parents Ram Chandra Panthee and Indira Panthee, wife Sudha Adhikari and brother Amod Panthee who always gave me love, patience, encouragement, support and their prayers which are valuable for the successful completion of my PhD study.

Abbreviations

AA, amino acid

A:P, acetic to propionic acid ratio

BW, bodyweight

CP, crude protein

DGGE, detergent gradient gel electrophoresis

DM, dry matter

DMD, dry matter digested

DMI, dry matter intake

DNA, deoxyribo nucleic acid

FDGL, Freeze dried garlic leaves

GC, gas chromatography

GC-MS, gas chromatography- mass spectroscopy

GO, garlic oil

ME, metabolizable energy

MH, mixed hay

N, Nitrogen

NDF, neutral detergent fiber

NH_3, ammonia

Rusitec, Rumen simulation technique

VFA, volatile fatty acids

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Chapter 1

General Introduction

1.1 Introduction

Livestock are essential to maintain human life as they can convert low quality, high fibrous materials into high quality protein sources such as milk and meat. Human consumption of meat and dairy products is however said to be a contribute a large fraction of environmental problem (FAO, 2006; Pelletier and Tyedmers, 2010). Currently, the livestock sector uses about 70% of all agricultural land and is responsible for about 15% of the total emission of anthropogenic greenhouse gas (Herrero et al., 2011; Morgavi et al., 2011; Bailey et al., 2014). The demand of food of animal origin is rising globally and is expected to increase by 70-80% between 2012 and 2050 (FAO, 2011; Bailey et al., 2014). Thus, a solution is needed to increase the current levels of production efficiency without expansion of farm land, and also with minimal agricultural wastes.

Methane is the byproduct of the anaerobic food chain, which is integral to ruminants to obtain energy from the feed. Methane is produced by a particular group of micro-organism called methanogenic archaea. Rumen microorganism ferment feedstuff to VFA, and is used by ruminants as energy source (approximately 80%); microbes themselves also are the source of microbial protein leaving the rumen and can account for much (60-85%), if not all of protein entering the small intestine (Hart et al., 2008). In the rumen, methanogens reduce the carbon dioxide to methane using mainly hydrogen as electron donor. Methane production is a natural process that prevents the accumulation of the hydrogen gas released by bacteria, protozoa, and fungi during the fermentation of the feeds, particularly the carbohydrate fraction. About two-thirds of the livestock methane is a result of enteric

fermentation (Moss et al., 2000) and comes mostly from the rumen (87%) and remaining from the intestine (Boadi et al., 2004). Although complete elimination of methanogens is not desirable, methane production can be modulated without affecting, or even improving, animal performance (Martin et al., 2010; Morgavi et al., 2010). The physical and chemical conditions of the rumen, influenced by the diet and the host animal, such as rate of passage, profile of VFA and pH also have an effect on methane production. As methane is produced by the methanogens, within the rumen, it follows that anything influencing these microbes will influence methanogens and thus methane production (Bhatta et al., 2007).

Liver glycometabolism of ruminants is different from that of monogastric animals. In the lactating dairy cows, glucose is supplied primarily by hepatic gluconeogenesis to maintain stable blood sugar (Li et al., 2013). Due to presence of foregut fermentative microbes, most carbohydrate ingested by ruminant is degraded to short chain fatty acids including acetate, propionate and butyrate, and remaining glucose is removed by cells of gastrointestinal tract prior to liver entry (Reynolds et al., 1991). Liver is thus responsible for glucose production in ruminants. Major glucose precursors of ruminant include propionate, glucogenic amino acids and lactate (Lemosquet et al., 2009a; Lemosquet et al., 2009b). Nonetheless, increased glucogenic precursors supply, via food or ruminal infusion, does not always elicit predicted increments in glucose production (Vargas-Bello-Pérez et al., 2008; Li et al., 2013). Therefore, a complex interaction of glucose metabolism exists in ruminant animals. Recently, under normal feeding conditions, different dietary ingredients were shown to affect glucose metabolism (Vargas-Bello-Pérez et al., 2008). Garlic is said to influence plasma glucose level in ruminants (Hodjatpanah et al., 2010; Kamruzzaman et

al., 2011; Kholif et al., 2012; Pirmohammadi et al., 2014; Anassori et al., 2015). Thus we tried to see the effects of plasma glucose metabolism in sheep fed freeze dried garlic leaves or garlic oil.

In recent years, there has been an increasing interest in the use of plant extracts to manipulate rumen fermentation, perhaps as a result of changes introduced within the European Union to prohibit the use of growth-promoting antibiotics, including monensin and related compounds in animal feed (McAllister and Newbold, 2008). There are a range of mechanism by which plant extract decrease methane production, including inhibition of ciliate protozoa, effects on fiber degradation and enhancement of propionate production. Direct inhibition of ruminal methanogenesis is another potential strategy, but few extracts have been confirmed as methanogens inhibitors. Among the plethora of many such plants, extensive research on garlic is carried out (McAllister and Newbold, 2008).

The origin of garlic (*Allium Sativum*) is reported to be as old as 5000 years ago when it was cultivated in Egypt and Mediterranean region and presently it is grown all over the world (Amagase et al., 2001). Garlic is commonly used for culinary purpose; however it is appreciated due to its therapeutic and medicinal properties, both in traditional and modern medicine. Garlic contains a complex mixture of many secondary metabolites which includes allicin ($C_6H_{10}S_20$), diallyl sulfide ($C_6H_{10}S$), diallyldisulfide ($C_6H_{10}S_2$) (Martins et al., 2016). Apart from its volatile compounds, garlic is also rich in vitamins (especially vitamins of B complex and vitamin C), antioxidants, flavonoids and minerals (especially P, K and Se) (Martins et al., 2016). Along with volatile oils (0.1-0.36%), it has also a good

source of protein (16-17%) and sulfur content in garlic varies from 0.5-3.7 per 100 g of dry matter (Kamra et al., 2012).

Garlic has several medicinal properties, but its anti-microbial activity against different pathogenic microbes is perhaps the most widely studied scientifically, using modern techniques of microbiology. Garlic acts as only mild antibiotics as compared to modern antibiotics, but added advantage of its use as antibiotics is those microorganisms do not develop a resistance against garlic as they against conventional antibiotics (Kamra et al., 2012). The antibiotic properties of garlic are direct result of allicin produced from raw and crushed garlic, which is destroyed by cooking or boiling.

Symbiosis between ruminant and their microflora instills ruminants with the unique advantage of being able to utilize non-protein sources of nitrogen as nutrients. The microbial protein that flows from the rumen to the small intestine provides the host with excellent sources of amino acid for synthesis of milk and meat. However, ruminant animals are relatively inefficient utilizers of dietary nitrogen. The efficiency of transfer of feed nitrogen into milk protein has been determined to be on average 24.7±0.14% (Hristov et al., 2005) with remaining nitrogen being lost to environment via feces and urine. Ruminal protozoa have a negative role on nitrogen utilization by ruminants. Protozoa engulf and digest large number of rumen bacteria thereby decreasing net microbial protein flow from rumen to duodenum (Ivan et al., 2000). Increased bacterial protein synthesis in the rumen due to defaunation could benefit the host by supplying additional amino acids for absorption (Benchaar et al., 2008). Nitrogen not used for growth, maintenance or milk

production contributes to soil, water and air pollution. As ruminally degradable nitrogen in excess of microbial requirements is largely lost to the animal, improving the efficiency of ruminal nitrogen is a primary goal in ruminant nutrition with far reaching production and environmental implications (Benchaar et al., 2008). Wanapat et al., (2008) found that feeding garlic powder at 80g/d with urea-treated rice straw could enhance nitrogen absorption and retention in cattle with decrease in ruminal protozoa populations. Reducing protozoa number often lowers rumen methanogenesis because ciliate protozoa have a symbiotic relationship with rumen methanogens (Newbold et al., 2015).

Tracer techniques based on isotopes are used by animal nutritionist to establish the values of feeds as well as protein, energy and mineral requirement of animals. The development of isotopic dilution procedures started in 1950s and 1960s made possible to measure the inflows into the body pools of the major energy yielding nutrients (glucose, acetate, 3-hydroxybutyrate and plasma non esterified fatty acids)(Annison and Bryden, 1999). Stable isotope tracers have advanced the field of metabolism by enabling the quantification of metabolic reactions in vivo. Stable isotopic tracers are easily disposable as well as they are also good for elements whose radioactive tracers are not available. The ease of disposal of stable isotopes is due to the fact that they do not undergo spontaneous decay with resulting emission that has biological effects. Stable isotopes are naturally occurring. Slightly more than one percent of all naturally occurring carbon is ^{13}C, thus the amount of isotopic carbon used in the experiment will have effect on the environment. An another advantage of stable isotope is that the use of selected ion monitoring with mass spectrometry enables definitive proof that the analyte has been isolated in absolutely pure form for the measurement of

stable isotope enrichment. If mass spectrometry is used to measure enrichment, then the ratio of tracer to trace is measured directly as opposed to the separate measurement of concentration and decays per minute (dpms) and the calculation of specific activity. The specific uses of stable isotopes are the use of ^{13}C and deuterium glucose for hepatic glucose production and ^{15}N for nitrogen metabolism in body. Nitrogen is the key element that defines amino acid and protein; however there is no radioactive isotope of nitrogen. Stable isotopes of carbon, hydrogen and nitrogen can produce the molecules of different molecular weight that retain the same metabolic functions known as isotopomers (Wolfe and Chinkes, 2005).

1.2 Objectives of the study

The thesis aimed to evaluate whether the use of dietary supplement, selected for their potential to reduce methane emission in ruminant could beneficially alter the production characteristics of sheep. Production characteristics were evaluated based on rumen fermentation characteristics, nitrogen utilization, methane emission, plasma glucose and amino acid kinetics. If there is no net advantage in production, it would be unlikely to be accepted by producers regardless of its potential to reduce methane emission. The first experimental diet included roughage vs. concentrate diets. Second experiment included various concentrations of freeze dried garlic leaves and bulb added to roughage diet on invitro rumen simulation technique (RUSITEC). Third experiment included addition of freeze dried garlic leaves to control diet and final experiment included addition of garlic oil on control diet.

The following objectives were established and experimentally tested via a series of experiments as described in Chapter 2-5.

1. To determine the effect of roughage based vs. concentrate diet on rumen fermentation characteristics, nitrogen utilization and microbial diversity analysis in sheep
2. To determine the effects of freeze dried garlic leaves and freeze dried garlic bulb on rumen fermentation, methane emission and dry matter digestibility using RUSITEC
3. To determine the effect of feeding freeze dried garlic leaves on rumen fermentation, nitrogen utilization, methane emission and plasma glucose kinetics in sheep
4. To determine the effect of feeding garlic oil on rumen fermentation, methane emission, nitrogen utilization, plasma glucose and amino acid kinetics in sheep

Chapter 2

Study on roughage vs. concentrate diet on methane production, rumen fermentation characteristics and microbial diversity in rumen

2.1 Introduction

Methane emission from livestock has gained a lot of interest among animal nutritionist and climate scientists due to the undesirable effect on environment as its contribution to global warming (Chandramoni et al., 2000; Moss et al., 2000). Methane emission is more likely to increase over next few decades as the population of livestock continues to grow to meet the growing demands for meat and milk primarily in developing countries (Thornton, 2010). Enteric methane emission is one of the main sources of GHC emission from livestock sectors and far most important emission source that can be targeted for mitigation within the ruminant production cycle.

The rumen, a large fore-stomach in ruminant, occupies over 70% of the total gastro-intestinal tract acts as a continuous fermentation vat and can hold volumes of about 15 L in small ruminants such as sheep and goats (Hoffman, 1993). Generally speaking, the output of methane emission form the rumen is influenced by many factors, such as diet, age, body weight, sex, species, genotyoes and environmental changes. The type of feed offered to ruminants can have a major effect on methane production (Martin et al., 2010). For example, the diet rich in fiber tends to produce more methane, but shows the reverse effect rich in starch. The forage to concentrate ratio has an impact on rumen fermentation and hence the acetate: propionate ratio. Given that propionate is probably the principal alternative H^+ sink after CH_4, it would therefore be expected that methane production would be less when high concentrate diets are fed. Ruminants fed high to moderate quality forages are estimated to lose about 6.5 % of gross energy intake (GEI) as methane (CH_4),

while those fed with high grain diets lose as little as 2% of the GEI (FAO, 2006; Herrero et al., 2011). In the rumen, the genus *Methanobrevibacter* is dominant and less affected by animals at inter or intra-species level, however the methane emission from animals intra species differs when feeding different diets, indicating that microbial diversity at species or strain level may respond to dietary treatments and possibly associated with methane emission (Chong et al., 2014). Thus, using the technique of Detergent Gradient Gel Electrophoresis (DGGE), we tried to evaluate microbial diversity in rumen with regard to roughage vs. concentrate diets.

In this chapter of the study, we examined the effect of feeding two iso-energetic diets with comprising of roughage only and roughage plus concentrate (40:60 ratio) in sheep in rumen fermentation characteristics, methane emission, nitrogen utilization, in addition, changes in rumen microbial diversity.

2.2 Materials and methods

2.2.1 Animals, dietary treatments and feeding

The handling of the experiment was carried out according to the guidelines established by the Animal Care Committee of Iwate University. Six crossbred (Suffolk × Corridale) sheep, all approximately 4 years of age and initial body weight of 53 ± 1 kg of body weight (BW) were used. Two dietary treatments were tested, one was mixed hay (Hay diet) of orchardgrass (*Dactylis glomerata*) and reed canarygrass (*Phalaris arundinacea*) and another was Concentrate (Conc. diet) consisting of mixed hay and concentrate at 60:40 ratio. Total diet allocated per day was 100 kcal ME/kg $BW^{0.75}$/day. Sheep were fed diet

once per day at 0900 h and had *ad libitum* access to water. The chemical treatments dietary composition is presented in Table 2.1.

Table 2.1 Chemical composition of dietary treatments

Ingredients	MH diet	Conc. diet
DM (g/kg)	86.4	87.8
CP (g/kg DM)	141	142
NDF (g/kg DM)	625	473
Ash (g/kg DM)	153	115
ME (Kcal/kg DM)	1.76	2.36

The experiment was carried out using a crossover design with a period of 21 days. The layout of the experiment is illustrated in Fig 2.1. The experimental period consisted of 14 days of adaptation to the diets and 7 days of sample collection. The sheep were housed in individual pens during the adaptation period and then moved to individual metabolic cages in a controlled-environment room at an air temperature of 23^{O}C and relative humidity of 70%, with light provided from 0800 to 2000 h. Body weight (BW) of animal were taken at start of experiment and on day 8, day 15, and after finishing the experiment. Nitrogen balance was carried out from day 15 to day 20 and VFA sample was taken on day 21.

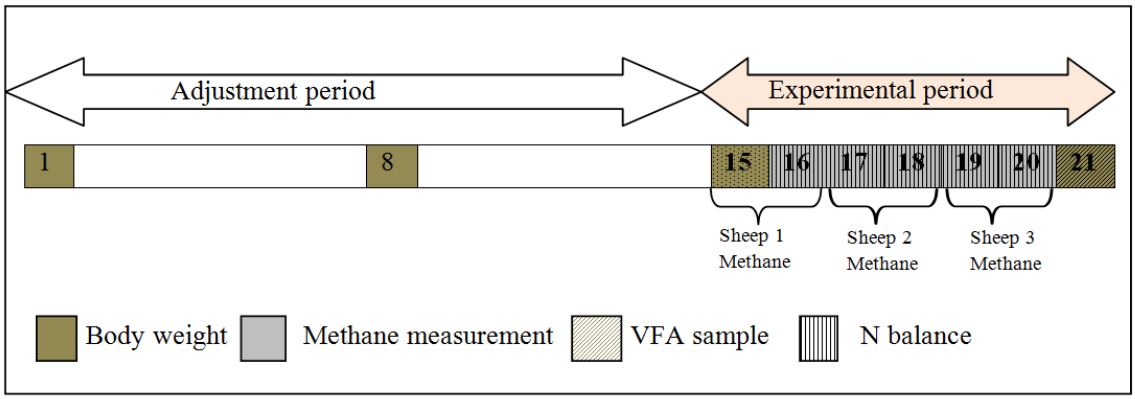


Fig 2.1 The experimental layout showing sampling protocol.

2.2.2 Collection of rumen fluid

On the 21st day of each sampling period, to characterize the ruminal fermentation patters, ruminal fluid (50 mL) was collected with a stomach tube inserted orally before feeding (0 h), 1.5 h, 3 h and 6 h after feeding. The pH values of rumen fluid were measured immediately with a pH meter (F-51, Horiba Ltd., Japan). The liquid fraction was separated by centrifugation at 8000 × g for 10 min at 4°C. An aliquot (5 mL) of ruminal fluid was used to measure the ruminal VFA concentration, and another 1 mL was acidified with 1 mL of 0.1 mol/L HCl to stop the microbial activity and prevent the loss of NH_3 concentration measurement. All samples were kept at -30°C.

2.2.3 Methane gas measurement

Sheep were accustomed to open circuit respiratory chambers (50 × 70 × 100 cm) prior to the experiment. Methane gas from rumen of each sheep released from oral cavity was analyzed for 2 days continuously using methane gas analyzer (VA 3000A, Horiba Ltd.,

Kyoto, Japan). Span gases of 0 ppm and 159 ppm methane were used to calibrate the methane analyzer before the experiment. 50 L/min of expired air inside the respiratory chamber was removed continuously using the pump and a portion was fed to methane gas analyzer. Background air methane gas concentration was measured and subtracted for each sheep. Feed was offered once per day and water was available *ad libitum*. The temperature, pressure and humidity were recorded simultaneously and converted into standard temperature and pressure conditions and thus final methane volume was standardized.

2.2.4 DNA extraction

Ruminal fluid collected before feeding (0 h), 3 h after feeding and 6 h after feeding was carried according to DNA extraction by bead beading process as described by Yu and Morrison (2004). Briefly, 0.5 g of mixed rumen sample was kept in 2 ml screw-cap tube with 1 ml of lysis buffer. The composition of lysis buffer was 500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA and 4% sodium deodecyl sulfate. A total of 0.4 g of sterile zirconium beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm) was added to lysis solution to increase the efficiency of lysis by mechanical disruption. The samples were then homogenized for 3 min using Taitec Beads Crusher μT-12 (Taitec Co., Japan) at 3200 r/min. They were further incubated at 70^{O}C for 15 min, with gentle shaking by hand every 5 min. After centrifuging at 16000 × g at 4^{O}C, 2 mL of supernatant was taken and further 300 μL of lysis buffer was added and re-homogenized. For precipitation of nucleic acids, 260 μL of ammonium acetate was added to lysate tube and incubated in ice for 5 min and centrifuged at 16000 × g at 4^{O}C for 10 min. The supernatant was distributed to two 1.5 mL

Eppendorf tubes and equal volume of isoproponal was added and incubated in ice for 30 min. It was then centrifuged at 16000 × g at 4^{O}C for 15 min. The pellet thus formed was washed with 70% ethanol and dried. The dried nucleic acid was then dissolved in 100 µL of TE (Tris-EDTA) buffer. The next stage was removal of RNA, protein and purification of extracted DNA. 2 µL of DNase-free RNase (10 mg/ml) and incubated at 37^{O}C for 15 min. Fifteen µL of proteinase K and 200 µL of Buffer AL from QIAamp DNA Stool Mini Kit was added, mixed and incubated at 70 ^{O}C for 10 min. Then, 200 µL of ethanol was added and mixed. Then it was transferred to QIAamp column and centrifuged at 16000 × g for 1 min. The flow through was discarded, 500 µL of Buffer AW1 (QIAamp DNA Stool Mini Kit) was added and again centrifuged for 1 min at room temperature. Flow through was discarded and 500 µL of buffer AW2 (QIAamp DNA Stool Mini Kit) was added and centrifuged for 1 min at room temperature. The column was dried by centrifugation again. Finally, 200 µL of buffer AE (QIAamp DNA Stool Mini Kit) was added and incubated at room temperature for 2 min. The sample was then centrifuged at room temperature for 1 min and elute was collected in fresh collection tube. The quality of eluted DNA was measured by absorbencies at wavelength of 260 nm and 280 nm using BioSpec-nano (Shimadzu Co. Japan). Samples with 260/280 absorbency ratio greater than 1.8 were of high quality suggesting RNA, proteins, or alcohols have been removed.

2.2.5 Polymerase chain reaction amplification of bacterial DNA

The 16S rRNA gene is used for phylogenetic studies as it is highly conserved between different species of bacteria and archea. In addition to highly conserved primer binding

sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature useful for identification of bacteria. Polymerase chain reaction (PCR) was carried out in Mycycler^{TM} thermal Cycler (Bio-Rad Laboratories Inc., USA). Forward primers included a GC-clamp at the 5'-terminus. A total of 40 µL PCR mixture containing 20 µL of EmeraldAmp PCR Master Mix, 4 µL of primers (357F-GC.518R diluted to concentration of 10 pmol/µL), 5 µL of pure water and 5 µL of template DNA (10 ng/µL) was prepared. The amplification was carried using the touchdown protocol: an initial denaturation for 5 min at 94^{O}C; 20 cycles of denaturation at 94^{O}C for 1 min, annealing at 65^{O}C for 1 min and extension at 72^{O}C for 1 min. The touchdown was carried out after cycle no. 2 was decreased by 1^{O}C every 2 cycles. 10 more cycles of amplification was carried out as follows: initial denaturation temperature of 94^{O}C for 1 min, annealing at 55^{O}C for 1 min and elongation at 72^{O}C for 1 min. The final elongation was carried out at 72^{O}C for 7 min. Finally, the samples were cooled down to 4^{O}C. The quality of resultant PCR product was checked in 2% agarose gel before denaturing gradient gel electrophoresis (DGGE) was carried out.

2.2.6 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis was carried out using DCode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories Inc., USA) on 130 V at 60^{O}C for 5 h. Two denaturing stock solutions of high density (60%) and low density (30%) were prepared as follows.

Table 2.2 Composition of denaturing stock solutions

Ingredients	Denaturing stock solution	
	30%	**60%**
40% Acrylamide/Bis solution, 37:5:1 (2.6%)	10 mL	10 mL
50X Tris-acetate EDTA buffer (TAE)	1 mL	1 mL
Formamid	6 mL	12 mL
Urea	6.3 g	12.6 g
dH_2O	Add to make 50 mL	Add to make 50 mL

Stock solution was then degassed using desiccators attached to vacuum pump for about 3 minutes. 135 µL of 10% APS and 6.8 µL of TEMED were added to each stock solution and mixed slowly. Thirty mL of each solution were kept in syringe and fixed on gradient delivery system which is attached by tubing to casting stand fixed with glass plates for gel preparation. Once the denaturation solutions were run through the system into DGGE plates, a comb was placed on top and gel was left to polymerize. Once the gel was polymerized, the cassette of gel with glass slides were taken out and fixed into main core of the electrophoresis unit into electrophoresis tank. About 7.0 L of 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8 with NaOH) was prepared and pre-warmed to 64^{O}C, wells of DGGE gels was washed by multiple pipeting. The PCR solution was pre-heated as follows: 95^{O}C for 5 min, 65^{O}C for 60 min, 45^{O}C for 30 sec followed by 35^{O}C for 30 sec and final temp at 25^{O}C. 3 µL PCR products were then mixed with 3 µL loading buffer

(70 %W/V glycerol). Mixed product (5 µL) is then loaded into respective wells in DGGE gels. Five µL of reference standard (Marker II; Nippon Gene Co, Japan) was loaded in side and middle of the gel containing 6 PCR products. The electrophoresis was then carried out at 60^{O}C for 5 h at 130 V.

When the electrophoresis was over, gel casting were removed and dissembled. The gel was then stained in SYBR gold solution (1X TAE Buffer 250 mL + SYBR gold 25 µL) for about 20 minutes. After staining the gel was placed in developing tray to be scanned and analyzed with AlphaImager Mini Imaging System (ProteinSimple, California, USA). The positioning of individual bands on the gel, relative to those of standard was identified.

Analysis of PCR-DGGE band patterns was accomplished using Gelcompar II (Ver. 6.5, Applied Maths, Inc., Cary, NC, USA). Diversity indices were calculated applying the following equations using band area as described by Patra and Yu, (2012).

Shannon Index (H) = $-\Sigma P_i \ln(P_i)$

Shimpson Dominance Index (λ) = $\Sigma (P_i)^2$

Evenness Index (e) = H/ln$S$

Where, P_i is the importance probability of the bands in a lane, calculated from ni/N, ni is the intensity of n^{th} band, and N is the sum of intensity of all bands, S is the total no of bands in the densitometer curve of each sample.

The diversity index is a qualitative measure that reflects how many different types are present in the dataset and simultaneously takes into account how evenly the basic entities are distributed among those types. The Shannon diversity index is used to characterize species diversity in a community. It accounts for both abundance and evenness of the species present. The proportion of species *i* relative to the total number of species (p_i) is calculated and then multiplied by the natural logarithm of this proportion (ln p_i). The resulting product is summed across species and multiplied by -1. Evenness index assumes the value between 0 and 1 and value 1 means complete evenness.

2.2.7 Microbial protein synthesis

For microbial protein synthesis, purine derivatives namely allantoin, xanthine plus hypoxanthine and uric acid from diluted urine samples were determined enzymatically following the procedure of Chen and Gomes (1995) using a spectrophotometer (V-630 BIO, JASCO, Japan). With this procedure, xanthine and hypoxanthine were measured together as uric acid after treatment of the urine sample with xanthine oxidase. For allantion determination, 1 mL of urine and standard samples were taken into 15 mL of glass tubes, mixed with 5 mL of distilled water and 1 mL of NaOH (0.5 mol/L) and 1 mL of phenyl hydrazine (0.023 mol/L) and the tubes were boiled again for another 7 min. After boiling for 7 min, the tubes were dumped into chilled ice for several min and added with 3 mL of concentrated HCl (11.4 N) and 1 mL of potassium ferricyanide (0.05 mol/L). After 20 min, the absorbance was read at 522 nm and allantoin concentration was concentrated was calculated (mg/L) based on the linear equation. For uric acid, 2.5 mL of urine and standard

samples were taken into 10 mL glass tubes and mixed with 1 ml phosphate buffer (KH_2PO_4, 0.67 mol/L). Two sets of standard and samples in duplicate were prepared. In one set, 150 µL of phosphate buffer and in other set 150 µl of uricase solution were added, mixed by vortexing and incubated in the water bath at 37 ºC for 90 min. Then the absorbance was read at 293 nm and uric acid concentration was calculated based on the standard equation. For xanthine plus hypoxanthine, 1 mL of urine and standard samples were taken into 10 mL glass tubes and added with 2.5 mL of phosphate buffer (KH_2PO_4, 0.2 mol/l) and 0.35 ml of L-histidine (4.3 mmol/l). Two sets of standard and samples in duplicate were prepared. In one set 150 µl of phosphate buffer and in other buffer and in other set 150 µL of xanthine oxidase solution were added, mixed properly and incubated in water bath at 37 ºC for 60 min. Then the absorbance was calculated based on standard equation.

The amount of microbial purine absorbed (X mmol/d) corresponding to the PD excreted (Y mmol/d) was calculated based on the relationship described by Chen et at. (1990) as follows:

$$Y = 0.84\ X + (0.15BW^{0.75}e^{-0.25X})$$

Where, 0.84 is the recovery of absorbed purine as PD in urine and $0.15BW^{0.75}$ represents the endogenous contribution of purine excretion. The calculation of X from Y based on the equation was made by means of the Newton-Raphson iteration process.

$$X(n+1) = X_n + \frac{f(x_n)}{f'(x_n)}$$

Where, $f(X) = 0.84\ X + (0.15BW^{0.75}e^{-0.25X}) - Y$

and the derivative of f(x): $f'(X) = 0.84\text{-}0.038\ 15BW^{0.75}e^{-0.25X}$

Given an initial value of $X_1 = Y/0.84$ to feed into the above equation to calculate X_2 and so on… X_{n+1} should have a constant value.

The equation for microbial nitrogen supply (MNS) is as follows

$$\text{MNS (gN/d)} = \frac{X\ (\text{mmol/d}) * 70}{0.83\text{x}0.116\ \text{x}\ 1000} = 0.727\ X$$

Where, 0.83 = digestibility of microbial purines, 70 = N content of purines (mg/mmol), and 0.116 = ratio of purine N to total N in mixed microbial biomass.

2.2.8 Chemical analysis

The chemical compositions of the diets were analyzed using the methods described by the Association of Official Analytics Chemists (AOAC, 1990). The neutral detergent fibre (NDF) content were determined according to Van Soest et al. (1991) using the Foss Analytical FibreCap system (FibreCap^{TM} 2021/2023, Foss, Sweden). Briefly, samples were weighed (1 g) in the capsules and extracted by boiling the capsules in the beaker containing NDF solution for 1 h. Then, the capsules were washed in a hot water (3 × 1 min), and then rinsed in methanol (2 × 1 min) and dried in the hot air oven (130 ^{O}C for 3 h). After drying, the capsules were weighed and then ignited in the muffle furnace (600 ^{O}C for 4 h). The composition of 1 L of NDF solution is listed in the Table 2.3

Table 2.3 Composition of NDF solution

Item	Amount (in 1 L solution)
Disodium ethylediamine tetra acetate (EDTA)	18.61 g
Sodium borate decahydrate	6.81 g
Sodium lauryl sulfate	30 g
Disodium hydrogenphosphate	4.56 g
Triethylene glycol	10 ml

The ruminal NH_3 concentration was determined as described by Weatherburn (1967) by mixing the ruminal fluid with the phenol and hypochlorite reagents and then incubating the sample in water bath at 37^{O}C for 20 min. The absorbance was measured by the spectrophotometer (V-630 BIO, JASCO, Tokyo, Japan) at 625 nm. The N content in the diets, urine and feces were analyzed using the Kjeldahl method with the Kjeltech system (Kjeltech 2100, Foss, Sweden). Briefly, samples were first digested by concentrated H_2SO_4 in presence of catalyst that promotes the conversion of NH_3 to NH_4^+. The NH_4^+ was then converted to NH_3 gas by distillation. Finally, the amount of trapped NH_3 was determined by titration with a standard solution and calculated by Foss Kjeltech system.

2.2.9 Calculation and statistical analysis

Results were presented as mean values with standard error of the mean. All data were statistically analyzed using analysis of variance with the MIXED procedure of SAS (1996). The least square means statement was used to test the effects of diet and time, with sheep as

random effect. Results were considered significant at the $P<0.05$ level, and a tendency was defined as $0.05 \leq P \leq 0.10$.

2.3 Results

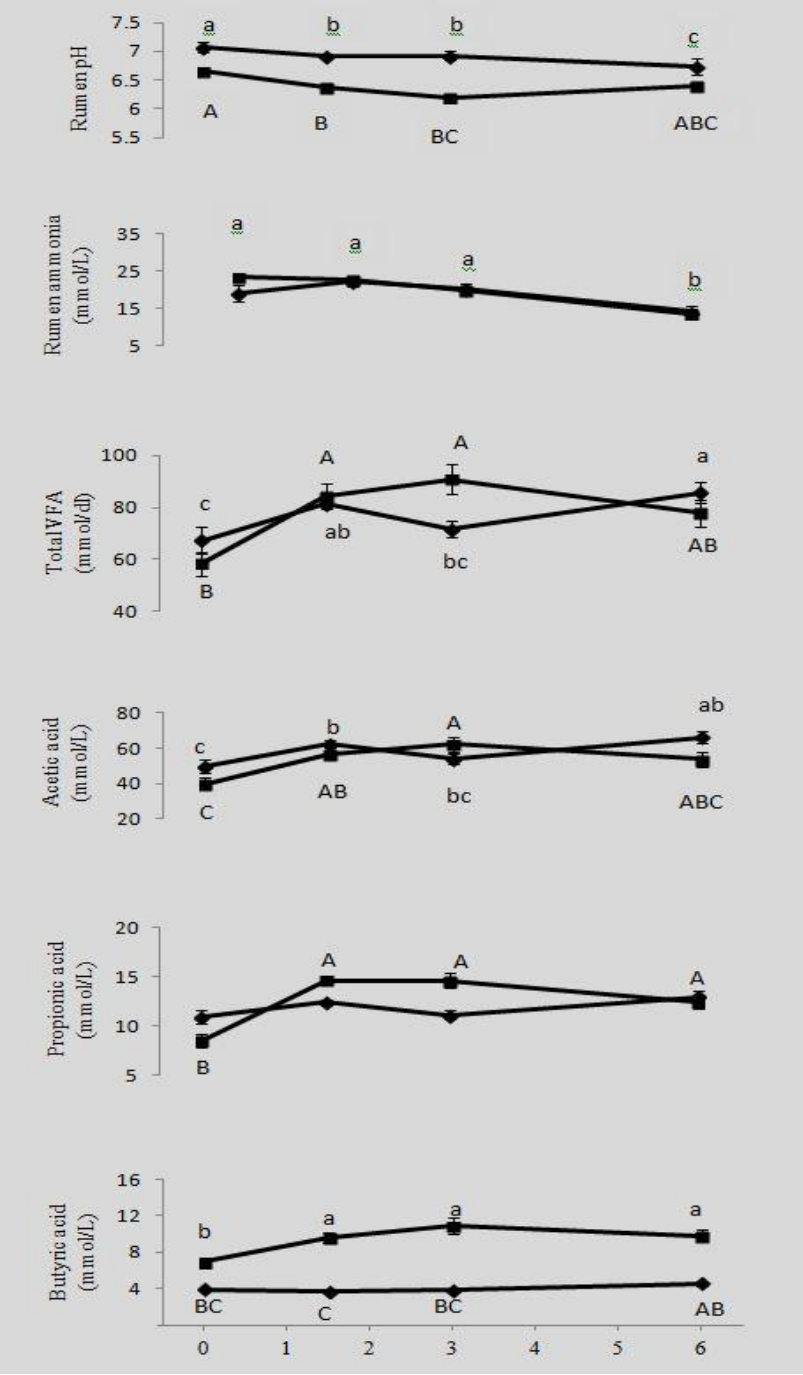
2.3.1 Rumen fermentation characteristics

2.3.1.1 Rumen pH

The result of average rumen pH is shown in Table 2.4 and the time course change in rumen pH in Fig 2.2. The rumen was lower in concentrate diet and there was treatment × time interaction.

2.3.1.2 Rumen ammonia

The result of rumen ammonia is presented in Table 2.4. There was no significant difference between the two diets and the ammonia concentration decreased at 6 h after feeding as shown in fig 2.2.



Effect	P value
Diet	0.0009
Time	0.0006
Diet × time	0.0099

Effect	P value
Diet	0.5579
Time	<0.001
Diet × time	0.1695

Effect	P value
Diet	0.7708
Time	0.0003
Diet × time	0.0135

Effect	P value
Diet	0.1768
Time	0.0003
Diet × time	0.0139

Effect	P value
Diet	0.1833
Time	<0.001
Diet × time	0.0015

Effect	P value
Diet	<0.0001
Time	0.0028
Diet × time	0.0046

Fig 2.2 Time course change in rumen pH, rumen ammonia, total and individual VFA in sheep fed Mixed hay (MH) diet (-♦-) and Concentrate (Conc.) diet (-■-). Values are expressed as mean ± SEM for n=6. Different letters (a,b,c and A,B,C) represent significant difference between time after feeding and small and capital letters represent significant differences in diets.

Table 2.4 Effect of dietary intake of MH diet vs. Conc. diets on rumen parameters in sheep[1]

Parameters	Treatment[2]		SEM[3]	*P*-value
	MH diet	Conc. diet		
No. of sheep	6	6	-	-
Body weight (kg)	53.1	52.9	1.32	0.85
pH	6.9	6.4	0.07	0.0009
Rumen NH_3 (mmol/L)	19.0	19.8	1.36	0.56
Rumen VFA (mmol/L)	76.5	77.9	3.73	0.77

[1] Values represent the mean values of 6 sheep.[2] MH diet, mixed hay of orchardgrass and reed canarygrass; Conc. diet, mixed hay plus concentrate (40:60 ratio) [3]SEM, standard error of the means

2.3.1.3 Rumen VFA concentration

The concentrations of individual volatile fatty acids and total fatty acid are presented in Table 2.5. The time course changes in individual volatile fatty acids are presented in Fig 2.2. Acetic and propionic acid did not differ between the diets. Butyric acid was lower in Conc. diet. Minor volatile fatty acids iso-butyric acid, iso-valeric acid did not differ between the diets however valeric acid was lower in MH diet.

Table 2.5 Effect of dietary intake of MH diet vs. Conc. diet in individual rumen VFA concentration and acetic to propionic acid ratio in sheep[1]

Parameters	Treatment[2]		SEM[3]	*P*-value
	MH diet	Conc. diet		
Acetic acid	57.9	53.2	3.11	0.18
Propionic acid	11.9	12.6	0.44	0.18
Butyric acid	4.1	9.3	0.44	<.0001
Iso-butyric acid	0.89	0.84	0.06	0.41
Iso-valeric acid	1.13	1.18	0.08	0.56
Valeric acid	0.50	0.68	0.03	0.004
A:P ratio[4]	4.84	4.28	0.15	0.01

[1] Values represent the mean values of 6 sheep.[2] MH diet, mixed hay of orchardgrass and reed canarygrass; Conc. diet, mixed hay plus concentrate (40:60 ratio) [3]SEM, standard error of the means.[4] Acetic acid to propionic acid ratio

2.3.2 Nitrogen utilization

The mean values of nitrogen in urine, feces, nitrogen absorption, nitrogen retention and nitrogen digestibility is presented in Table 2.5. The nitrogen intake was higher in MH. diet than conc. diet because the both diet were maintained to posses similar energy content and MH diet being lower energy, more amount was added to diet resulting in higher nitrogen intake. The fecal and urinary nitrogen excretion was lower in Conc. diet but due to lower nitrogen content in Conc. diet, nitrogen absorption as well as nitrogen retention came to be same. The nitrogen digestibility was higher in Conc. diet.

Table 2.6 Effect of dietary intake of MH diet vs. Conc. diet on nitrogen utilization in sheep[1]

Parameters	Treatment[2]		SEM[3]	*P*-value
(g/kg $BW^{0.75}$/d)	MH diet	Conc. diet		
Nitrogen intake	1.26	1.02	0.004	<.0001
Fecal Nitrogen	0.46	0.26	0.02	<.0001
Urinary Nitrogen	0.62	0.52	0.03	0.04
Nitrogen absorption	0.80	0.76	0.02	0.11
Nitrogen retention	0.18	0.23	0.04	0.27
Nitrogen digestibility (%)	63.4	74.3	1.65	0.002

[1] Values represent the mean values of 6 sheep.
[2] MH diet, mixed hay of orchardgrass and reed canarygrass; Conc. diet, mixed hay plus concentrate (40:60 ratio)
[3]SEM, standard error of the means

2.3.3 Microbial protein synthesis

The mean values of microbial protein synthesis, microbial protein supply and purine derivatives are presented in Table 2.7. Purine derivatives namely allantoin, hypoxanthine and xanthine, and uric acid were higher in Conc. diet. The total purine derivatives were higher in Conc. diet and thus the microbial protein synthesis was also higher in Conc. diet compared to MH diet.

Table 2.7 Effect of dietary intake of MH diet and Conc. diet on purine derivatives and microbial protein synthesis in sheep[1]

Parameters	Treatment[2]		SEM[3]	*P*-value
(mmol/kg $BW^{0.75}$/d)	MH diet	Conc. diet		
Allantoin	0.34	0.47	0.008	0.0002
Hypoxanthine and Xanthine	0.05	0.06	0.004	0.04
Uric acid	0.03	0.04	0.0008	0.0003
Total purine derivatives	0.43	0.57	0.0009	<.0001
Microbial protein synthesis	0.49	0.67	0.01	0.0001

[1] Values represent the mean values of 6 sheep.[2] MH diet, mixed hay of orchardgrass and reed canarygrass; Conc. diet, mixed hay plus concentrate (40:60 ratio). [3]SEM, standard error of the means

2.3.4 Methane emission

The total methane concentration during the 24 h period did not differ between the diets. The total methane emitted during the 24 h period was 32.4 L/d in MH diet and 34.4 in Conc. diet. The methane emission based on dry matter intake was higher in Conc. diet and based on dry matter digested tended to be lower in Conc. diet.

Table 2.8 Effect of dietary intake of MH diet and Conc. diet on methane emission in sheep[1]

Parameters	Treatment[2]		SEM[3]	*P*-value
	MH diet	Conc. diet		
Methane (L/d)	32.4	34.4	1.41	0.22
Methane (L/$BW^{0.75}$)	1.60	1.76	0.06	0.07
Methane (L/kg DMI)	28	40	1.33	0.0008
Methane (L/kg DMD)	50	47	1.44	0.09

[1] Values represent the mean values of 6 sheep.[2] MH diet, mixed hay of orchardgrass and reed canarygrass; Conc. diet, mixed hay plus concentrate (40:60 ratio).[3]SEM, standard error of the means

2.3.5 Microbial diversity analysis

The image of DGGE lanes is shown in Fig 2.3 There was not such a distinct band present in one of these groups which means that the microbe's types were almost similar however their relative abundance was different in two diets as shown by different band intensities. The microbial diversity indices such as Shannon index, Dominance index and Evenness index is presented in Fig 2.4 and did not differ between diets and also in different time period analyzed.

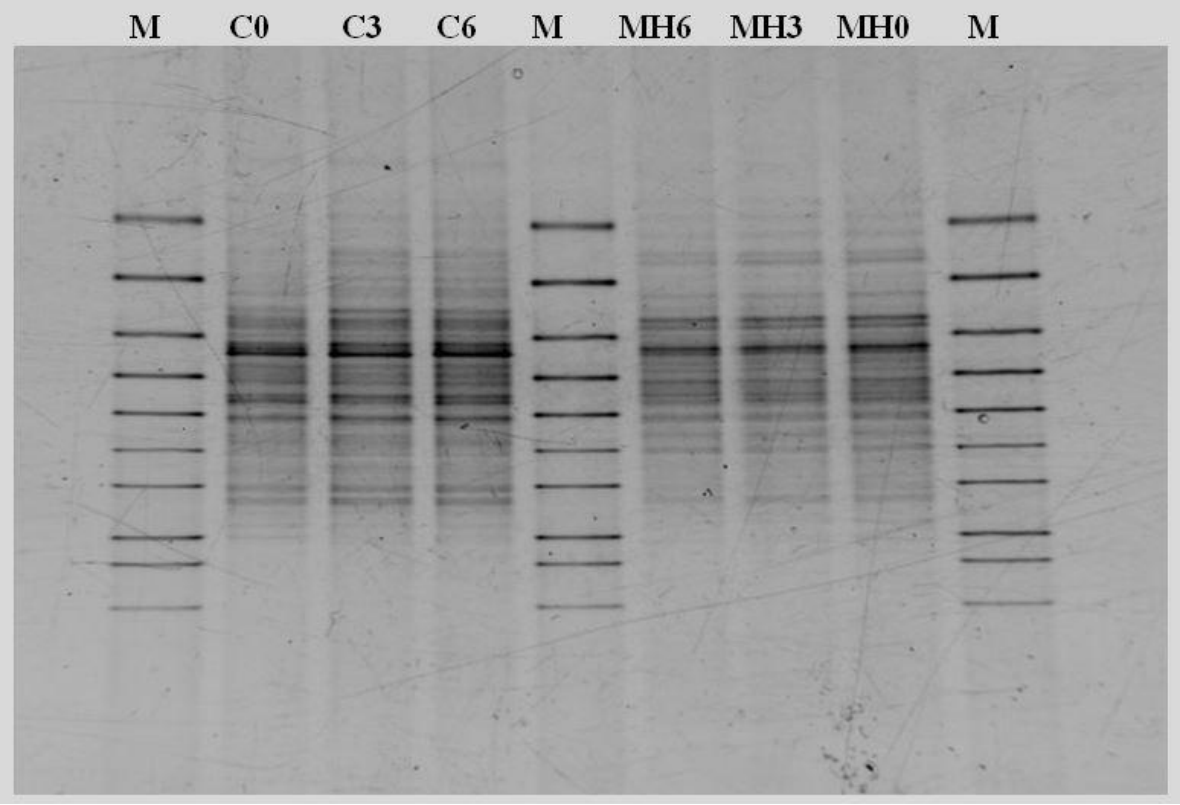


Fig 2.3 DGGE analysis showing profiles of 16S rRNA gene fragments from MH diet and Conc. diet at 0 h, 3 h and 6 h after feeding. M-DGGE reference marker II lane, C0-Concentrate diet 0h, C3-Concentrate diet 3h, C6-Concentrate diet 6h, MH0- mixed hay diet 0h, MH3- mixed hay diet 3 h and MH6-mixed hay diet 6 h.

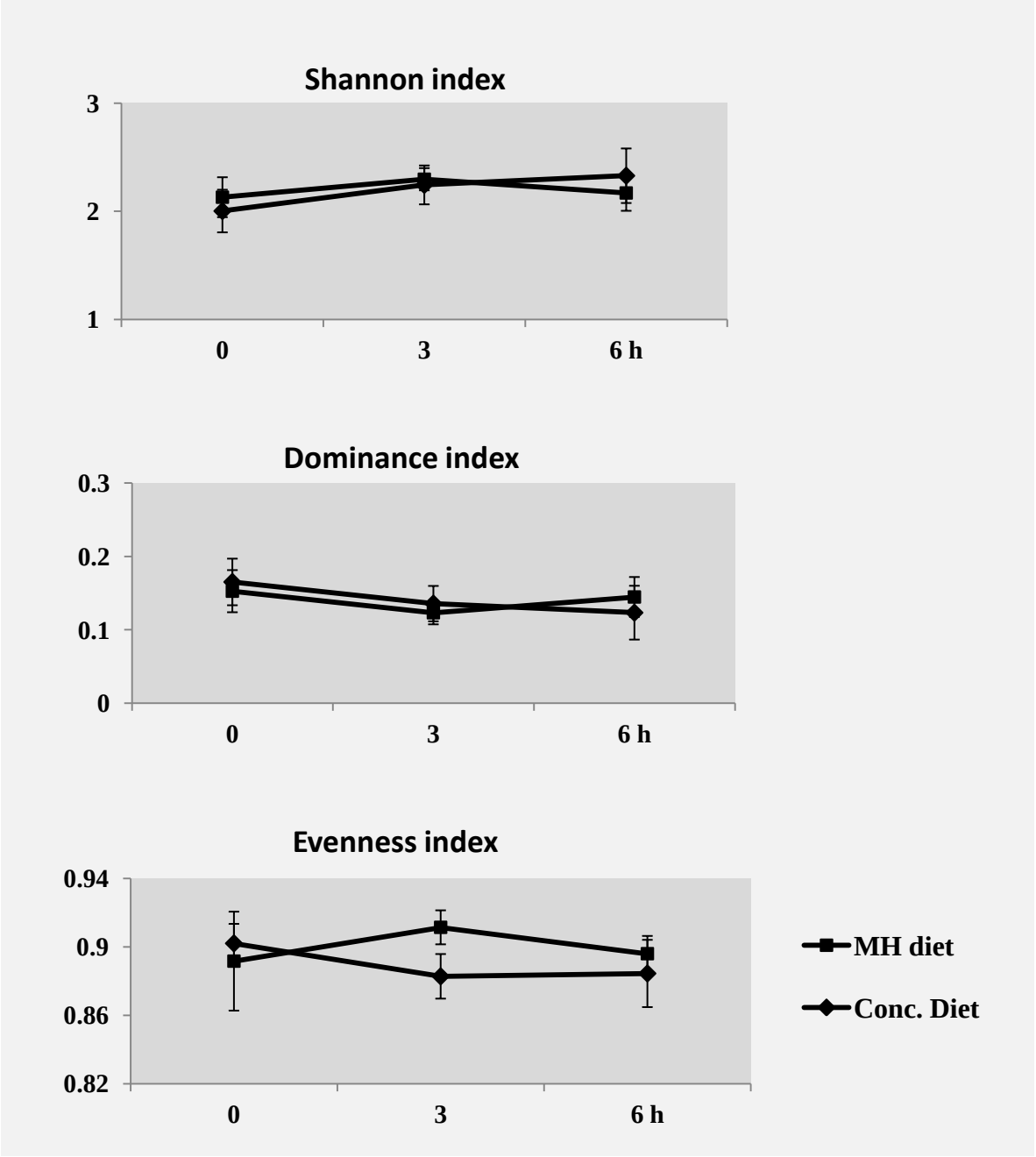


Fig 2.4. Effect of dietary intake of MH diet (-■-) vs. Conc. diet (-♦-) on diversity index in sheep. The values are expressed as the mean ±SEM for n=6.

2.4 Discussion

2.4.1 Rumen fermentation characteristics

Ruminal fermentation of ingested feed plays a major role in the supply of energy for metabolic functions in ruminants. Rumen pH in both the diets were within the normal range (5-7.5), however it was significantly lower (P=0.0009) in Conc. diet. This might be partially related starch portion of the Conc. diet which might be rapidly fermented compared to MH diet. This study is consistent with previous studies by Carro et al., (2000), Na et al., (2013) Chen et al., (2015),where rumen pH linearly decreased as concentrate in the diet was included .

The effect of forage to concentrate ratio on NH_3-N, total VFA and individual VFA concentration in ruminants have been investigated widely, but the results were inconsistent. In accordance with the previous researchers (Carro et al., 2000; Agle et al., 2010; Chen et al., 2015) our results showed that addition of concentrate in diet did not influence rumen $NH_{3,}$ and total VFA in the rumen. Several possible explanations exist for this difference. First, it might be due to rumen ecosystem in sheep being able to adapt to change in diet. Similarly, sheep fed roughage diet might have near similar degradation rate between protein and carbohydrate, which then increased the growth of ruminal bacteria compared with Conc. diet as Russell et al., (1992) explained. Moreover, NH_3 and total VFA had no difference in treatment, indicating that no acute change occurred within the rumen ecosystem. Regarding the individual VFA, feeding Conc. diet resulted in higher values on butyric acid and Valeric acid. Butyrate is converted in the rumen wall to a ketone body, β-

hydroxyl butyric acid (BHBA), which is very important energy source for most tissue. The molar proportion of butyrate has been reported to increase with addition of soluble sugars and concentrate feed (Reis and Combs, 2000). Sun et al., (2015) found that partially replacing cornstarch in concentrate diet with sucrose increased butyric and valeric acid, suggesting sucrose in place of starch increases butyrate and valeric acid concentration. Ribeiro et al., (2005) suggested that higher butyrate concentrate might result from change in fermentation pathway to accommodate the higher flux of hydrogen from rapidly fermented sugar source. Higher butyric acid and valeric acid in Conc. diet might be due to shift in bacterial population metabolizing feed substrate to products with greater usable energy values. Similarly, Brossard et al., (2004) suggested that increase in entodinimorph protozoa numbers promote butyrate production, which might also be one plausible reason of higher butyrate in our study.

Rumen NH_3-N is the end product fermented from the feed protein and other nitrogenous compounds in rumen, which function as raw material for rumen bacteria to synthesize microbial proteins. NH_3-N concentration in the rumen can vary greatly depending upon diet, time of feeding and feeding frequency, animal and other factors. Under normal feeding conditions, a large proportion of dietary protein will pass through ammonia pool before it is utilized for microbial protein synthesis in the rumen. Ammonia utilization by microbes in the rumen is intrinsically related to rate of release and carbohydrate availability (Hristov et al., 2005; Jasim et al., 2015). In this experiment no change in NH_3-N concentrate indicates both diets were similar with regard to ammonia utilization.

On higher concentrate diets, the availability of fermentable energy will be more which helps rumen microbes to capture NH_3-N leading to increased digestibility and absorption (Malisetty et al., 2014). In our experiment, nitrogen absorption and nitrogen retention was similar but fecal and urinary nitrogen loss was lower in Conc. diet. However, the nitrogen digestibility was higher in Conc. diet indicating the higher efficiency of the diet. Variable level nitrogen intake in the two diets might be responsible for similar nitrogen retention and absorption as in Conc. diet being high in energy value, the nitrogen intake got lowered as our diet was isoenergetic. Our results agree with Carro et al., (2000); Ramos et al., (2009), where nitrogen retention did not differ in diet fed high concentrate diet in sheep.

2.4.2 Nitrogen utilization

Optimizing the utilization of dietary nitrogen requires good knowledge of partitioning of feed nitrogen between productive protein and excretion products (urinary and fecal N losses). The nitrogen intake in our study was lower for Conc. diet compared to control diet. This was because concentrate diets are generally high in energy and we fixed the same metabolic energy level of 100kcal/kg$BW^{0.75}$/day for both diets. Although the fecal nitrogen excretion was lower for Conc. diet, the nitrogen absorption for both diets was similar. This was because of difference in level of nitrogen intake in two diets. The urinary nitrogen differ significantly in both diets however the nitrogen retention did not differ in two diets. From the viewpoint of nitrogen utilization in both diets, we can say that Conc. diet was better in utilization of nitrogen sources efficiently compared to MH diet. This can also be

supported by the fact that nitrogen digestibility in Conc. diet was higher compared to MH diet.

2.4.3 Microbial protein synthesis

Microbial protein has a good balance of essential amino acid and thus its synthesis in rumen should be optimized. Optimization of microbial protein synthesis should increase the efficiency of nitrogen utilization and reduce nitrogen urinary excretion. The urinary excretion of purine derivatives (allantoin, uric acid and xanthine and hypoxanthine) has been suggested as an estimator of the microbial protein flow to the duodenum in the ruminant animals (Gonda et al., 1996). Urinary purine derivatives appear to be reliable non-invasive method for estimation of microbial protein in ruminants (Chen and Gomez, 1992; Gonda et al., 1996). The efficiency of microbial protein synthesis in Conc. diet was higher and this might be due to readily available source of energy provided by the easily fermentable carbohydrate present in the Conc. diet. Pathak (2008), Ramos et al., (2009) and Nalini Kumari et al., (2012) reported that sheep fed a diet composed of mixture of hay and concentrate had a greater microbial growth and thus microbial protein synthesis in the rumen compared to those fed hay or concentrate alone. The reason was because forages supply nitrogen as highly degradable protein or non-protein nitrogen, concentrates slowly supply nitrogen as peptides and amino acid required for microbial protein synthesis.

2.4.4 Methane emission

Methane emission (L/d) in MH diet and Conc. diet did not differ in our studies. Our result seems to agree with Shibata et al., (1992), Chandramoni et al., (2000) where methane

production measured by open circuit respiration calorimetric technique in sheep fed concentrate diet up to 70 % did not differ with roughage diet. The main component affecting methane production is the type of carbohydrate and relative rate of fermentation (Chandramoni et al., 2000). Methane production in the rumen is directly associated with the formation of hydrogen, therefore methane production can be lowered by depriving methanogens with hydrogen. Higher dietary content of fiber increases the production of acetate, whereas diets with higher content of starch favor propionate formation as observed in our study by lower acetic acid to propionic acid ratio. But this lower acetic to propionic acid ratio could not be validated in methane emission in our study; this might be associated with multiple reasons. First it may be due to, in part limitations of the experimental protocol as methane concentration measurement of inflow air was not carried out in our experiment. Secondly, the molar proportions of ruminal VFA do not necessarily represent the proportion in which they were produced (Aguerre et al., 2011) but rather balance between production and absorption. Similarly presence of energy from carbohydrate fraction might have increased the population of rumen micro-organism in Conc. diet compared to MH diet which in turn increased digestion and thus methane emission. Methane emission (L/kg DMI) was higher in Conc. diet in our study as the concentrate fraction of diet had more energy compared to roughage diet and thus feeding iso-energetic diet lowered the dry matter intake in Conc. diet. Similarly, lower tendency of methane emission (L/kg DMD) in Conc. diet can be attributed to higher pass rate in rumen as roughage diet tends to be bulkier and retain more time in rumen resulting in higher methane emission compared to concentrate diets. Furthermore, Shibata et al., (1992) also mentioned

that when the feeding level is less than 1.5 times the maintenance requirement, no effects due to nature of carbohydrate components in methane observation can be seen. However, been said that if total methane emission is calculated based on dry matter intake, there would be error as Conc. diet being had lower intake but similar methane emission as roughage diet.

2.4.5 Microbial diversity indices

Microorganism plays an important role in all facets of rumen fermentation and are also highly responsive to diet, age and health of host animal (Nagaraja and Titgemeyer, 2007; Petri et al., 2012). The microbial diversity (Shannon, Dominance and Evenness index) of the rumen was not affected both by diet and time in our study. In a healthy growing ruminant species, diet composition is the most important driver of hierarchical structural changes in bacterial populations (Petri et al., 2012). It is generally considered that increase in available carbohydrate results in increased microbial growth which results in increase in fermentation (Nagaraja and Titgemeyer, 2007). According to the DGGE, band profiles did not differ significantly between diets in our study. Similar study carried out by Kocherginskaya et al., (2001)showed that the no of bands and places were similar in DGGE but diversity of species were high in concentrate diet when they carried out further band analysis. Our study did not analyzed the genetic structure of the bands thus the effect was not observed. DGGE methodology although is rapid and repeatable method for identification of diversity of micro-organisms, this method has its limit that co-migration of DNA along the gels results bands with multiple bacterial species have on a same band.

Furthermore, the bands were not sequentially analyzed for DNA nucleotides sequences present, thus the difference could not be distinctly observed.

2.5 Conclusion

The overall effect of replacing forage diet with concentrate did not have effect on rumen fermentation characteristics such as ammonia and rumen VFA except rumen pH was lower in Conc. diet. However, the ratio of acetic to propionic acid was lower in Conc. diet; this may indicate the relative efficiency of Conc. diet. The acetic propionic acid ratio however was not translated into total methane production in 24 hour, this might be because after six hours the methane production might have increased. Although the nitrogen retention did not differ in two diets, however the difference in nitrogen intake means that nitrogen retention was relatively better for Conc. diet. This was also supported by the fact that nitrogen digestibility was higher in Conc. diet. The microbial protein synthesis was also higher in Conc. diet. The methane emission per day was similar on per day basis however in terms of dry matter intake it was higher in Conc. diet and dry matter digestibility, the methane emission was lower in Conc. diet. This was because the energy values were higher on dry matter basis for Conc. diet. The microbial diversity based on DGGE analysis did not show difference in diet. In overall, the Conc. diet was better in terms of nitrogen utilization and microbial protein synthesis, which if could translate into productive performances such as meat, milk and wool growth; we can say feeding Conc. diet would be advantageous in decreasing methane per unit of production.

Chapter 3

Effect of freeze dried garlic leaves and bulb on rumen fermentation and methane production in in-vitro rumen simulation technique (Rusitec)

3.1 Introduction

Due to the increased concerns of greenhouse gas emission and the restriction on the use of the antibiotics in the livestock industry, secondary plant metabolites have received a noticeable attention in the recent years. Earlier, numerous chemical additives to ruminant feed have been used to inhibit methanogenesis (Bozic 2009, Patra 2012, Hirstov 2013). These chemicals however are toxic to hosts or exhibit only transient effects on methanogenesis, and thus natural products are seem to more acceptable in this regard (Patra 2012, Hirstov 2013). Plants that contain bioactive products, such as essential oils, tannins can protect themselves from microbial and insect attack. Among the plethora of plants with secondary metabolites, Garlic is one such compound which is widely accepted as human medicine since prehistorically times. Garlic contains complex mixtures of many secondary metabolites, which include allicin ($C_6H_{10}S_2O$), diallyl disulfide ($C_6H_{10}S_2$) and allyl mercaptan (C_3H_6S). In addition to that it is a rich source of sulfur potassium, phosphorous, magnesium, sodium and calcium (Kamra et al., 2012).

The objective of this study were to see the effects of various doses of raw garlic and garlic leaves for manipulation of rumen fermentation and see it effects on pH, volatile fatty acid production, ammonia production and methane production using rumen simulation technique (Rusitec).

3.2 Materials and methods

3.2.1 Rumen fluid donor animal and diets

Animals used in the experiment were handled following the protocols provided by Animal Care Committee of Iwate University. Two fistulated sheep of 46.5±0.8 kg body weight were used in the experiment as donor of rumen fluid. Sheep were fed with mixed hay and concentrate at ratio of 80:20. Sheep were fed maintenance diet of 120 kcal ME/$kg^{0.75}$/day once daily every morning and water was available *ad libitum*. Mixed hay consisted of orchardgrass and reed canarygrass at 60:40 proportions. Commercial concentrate was obtained from Chubu Shiryo Co. Ltd., Japan. The rumen fluid was collected every morning before the feed was offered to animals.

3.2.2 Preparation of freeze dried garlic leaves and garlic bulb

Fresh garlic bulb (GB) and garlic leaves (GL) were obtained from commercial garlic farm (Sawada Farm, Aomori, Japan). Raw garlic bulb were then sliced about 0.5 cm and garlic leaves were chopped about 2-3 cm in length and freeze dried (EYELA freeze dryer FD/5N, Tokyo Rikakikai Co., Ltd, Tokyo, Japan). These freeze dried samples were further grinded and passed through 1 mm screen. .

3.2.3 Invitro incubation in Rusitec

Rumen fluid was obtained from two sheep before morning feeding and pooled. Mixed ruminal microorganism cultures were maintained in semi continuous flow fermenters known as Rumen Simulation Technique (Rusitec) for 9 days. It consisted of 8 vessels with 800 mL effective volume operated following the procedure of Czerkawski and Breckenridge (1997) and described in detail by Kajikawa et al. (2003), with some modifications. Briefly, rumen fluid was collected in a thermometer previously filled with

warm water at 39^{o}C. Rumen samples were then brought to lab quickly and filtered through four layers of cheesecloth and stored under anaerobic condition using CO_2 gas. On day 1, each vessel was inoculated with buffered rumen fluid and one nylon bag (100µm pore size) filled with 70 g of solid rumen content and other with respective 15 g of substrate dry matter supplied in nylon bag. Artificial saliva (McDougall, 1948) was continuously infused using a peristaltic pump adjusted to maintain a liquid dilution of 3%/h. Each vessel was fed daily at 0900 with new sample in nylon bag and after 2 days digestion the nylon bags were removed.

The basal diet, without any supplementation was used as negative control, henceforth referred as Control diet. As positive control, the Control diet was supplemented with freeze dried garlic powder and freeze dried garlic bulb powder were used.

3.2.4 Methane gas measurement

Gas produced from each fermentation vessel was measured from Teflon bag attached to collection vessel in the bottom of the Rusitec machine. Formalin was added to collection vessel to inhibit the microbial activity. Formalin was added initially to the collection vessel such that final concentration of formalin solution would be around 1% of total effluent volume collected. The volume of gas collected in Teflon bag was measured using a dry test gas meter model DC (Shinagawa Co., Ltd, Japan).

A portion of total gas collected in teflon bag was used for methane concentration analysis using gas chromatography (HP-5890, Hewlett Packard, USA) using a HP-PLOT Q, 0.53

mm × 30 m (Aligent Technologies, USA). The initial oven temperature was 100^{O}C and final temperature was 110^{O}C increased at rate of 10^{O}C/min. Flame ionization detector at 250^{O}C was used to measure signal amount and data was plotted in HP-3329A Integrator (Hewlett Packard, USA) . Methane calibration was done by using 10% standard methane gas. 1 µL of sample methane gas was injected in triplicate and data reanalyzed when values differed more than 5% of mean values.

3.2.5 Rumen fermentation parameters and invitro dry matter digestibility analysis

About 10 ml of rumen fluid was obtained at initial (0 h), 3 h and 6 h from the incubation vessel via sample collection outlet. Total VFA produced during 24 h were calculated using the collection vessel sample. Rumen pH, ammonia and VFA were analyzed at described earlier in Chapter 2.

Nylon bags after 2 days of fermentation in the vessel were taken out and washed lightly in washing machine and dried in hot air oven at 60^{O}C for 48 hours. Dry matter disappeared after drying the sample was used to measure the invitro dry matter disappearance of diets.

3.2.6 Statistical analysis

Results were presented as mean values with standard error of mean. All data were analyzed using mixed procedure of Statistical Analysis System Institute, Inc (SAS, 1996). Results were considered significant at the $P<0.05$ level, and a tendency was defined as $0.05<P\leq0.10$.

3.3 Results and Discussion

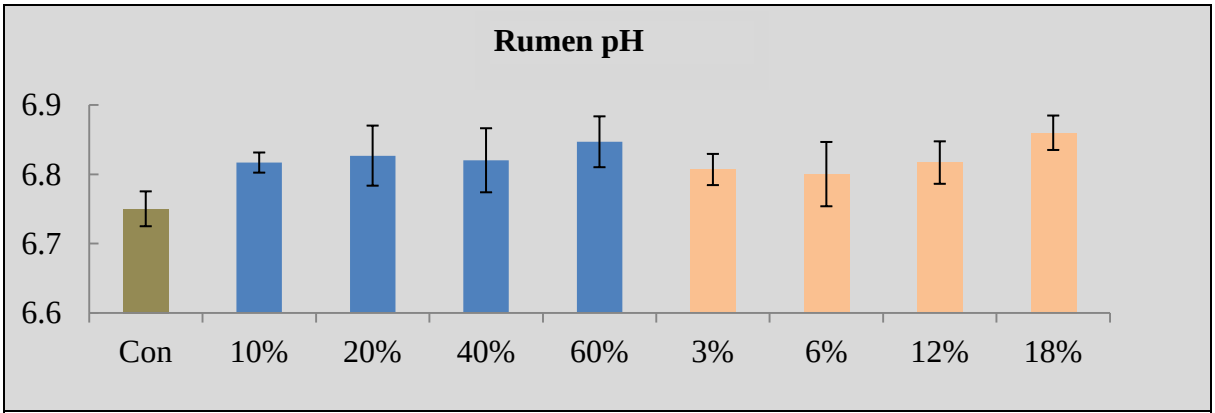


Fig 3.1
Effect of addition of garlic leaves or garlic bulb in rumen pH on invitro fermentaion Rusitec. The values are expressed as mean ± SEM. Con. diet = mixed hay 15g DM; 10%, 20%, 30%, 40%, 60% = Con. diet plus freeze dried garlic leaves added at 10%, 20%., 30%, 40%, 60% DM respectively and 3%, 6%, 12%, 18% = Con. diet plus freeze dried garlic bulb added at 3%, 6%, 12%, 18% DM respectively.

The effect of addition of garlic leaves and garlic bulb in rumen pH is shown in figure 3.1. No significant differences were found in rumen pH among the group. Several experiments show that the addition of garlic leaves, bulb, garlic oil or its active components does not influence the rumen pH in both invitro and invivo experiments. Ruminal pH also did not change when 300mg/l of garlic oil was incubated in Rusitec by Soliva et al., 2011. Similarly, an active component found in garlic allyl isothiocynate at 75 mg/L also did not influence ruminal pH in same experiment (Soliva et al., 2011). No effect on rumen pH was also observed by Kamruzzaman et al., 2011 on rumen pH when sheep were fed garlic stem and leaves silage at 10% feed replacement. Klevenhusen et al., 2011 carried out an experiment in sheep using garlic oil at 5 g/kg DM and diallyl disulfide at 400 g/kg and also found no difference in rumen pH.

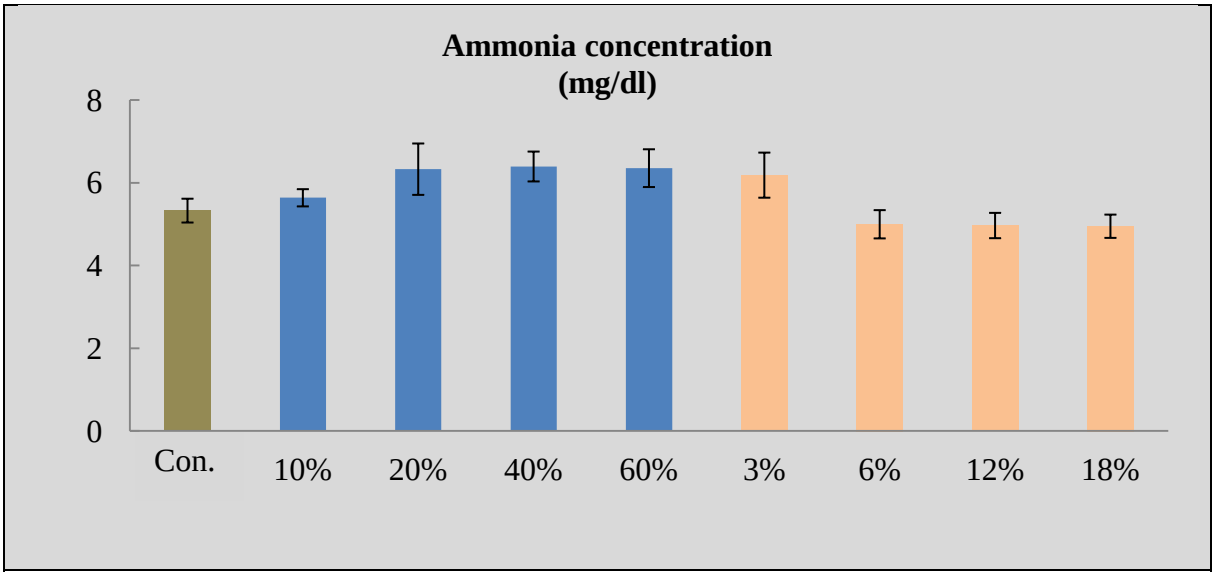


Fig 3.2
Effect of addition of garlic leaves or garlic bulb in ammonia concentration on invitro fermentation Rusitec. The values are expressed as mean ± SEM. Con. diet = mixed hay 15g DM; 10%, 20%, 30%, 40%, 60% = Con. diet plus freeze dried garlic leaves added at 10%, 20%., 30%, 40%, 60% DM respectively and 3%, 6%, 12%, 18% = Con. diet plus freeze dried garlic bulb added at 3%, 6%, 12%, 18% DM respectively.

The effect of addition of garlic leaves and garlic bulb in rumen ammonia concentration in Rusitec is shown in figure 3.2. The ammonia concentration did not differ among the experimental groups. Busquet et al., 2005a also found that addition of garlic oil to dual flow continuous culture at 31.2 mg/L and 312 mg/L did not change the ammonia concentrations. Furthermore garlic oil also did not change rumen ammonia when garlic oil was added to rusitec at rate of 3, 30 mg/L but at 300 and 3000mg/L ammonia concentration decreased in Rusitec (Busquet et al., 2006). Pure compounds from garlic such as diallyl disulfide and lovastatin when fed to sheep at rate of 4 g and 80 mg per kg dry matter respectively did not influence rumen ammonia concentration in sheep. Klevenhusen et al.,

2011 however found decrease in ammonia concentration when garlic oil was fed at 5g/kg dietary dry matter but not in 2g/kg diallyl disulfide.

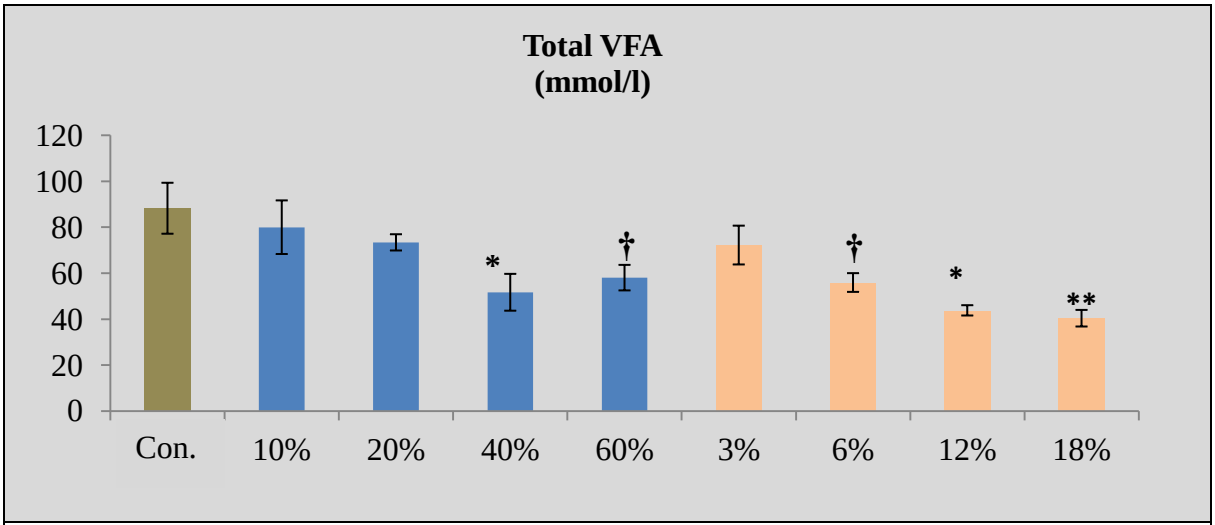


Fig 3.3
Effect of addition of garlic leaves or garlic bulb in total VFA on invitro fermentation Rusitec. The values are expressed as mean ± SEM. Con.diet = mixed hay 15g DM; 10%, 20%, 30%, 40%, 60% diet = Con. plus freeze dried garlic leaves added at 10%, 20%., 30%, 40%, 60% DM respectively and 3%, 6%, 12%, 18% diet = Con. plus freeze dried garlic bulb added at 3%, 6%, 12%, 18% DM respectively. * Means differ from control diet ($p<0.05$). † Means differ with control diet($p<0.10$)

Table 3.1 Effect of addition of garlic leaves and bulb on individual VFA on invitro fermentation Rusitec.

	Treatment									P value
	Con. diet	**10%**	**20%**	**40%**	**60%**	**3%**	**6%**	**12%**	**18%**	
Total VFA	88.20	79.95	73.36	51.62*	57.99*	72.18	55.88†	43.77*	40.34*	0.001
Acetic acid	49.46	49.87	43.37	29.71*	32.80	37.16	29.35*	25.47*	22.51*	0.001
Propionic acid	22.07	20.97	17.80	12.96	16.04	22.06	16.61	11.68†	10.88†	0.015
Iso-butyric acid	0.63	0.58	0.48	0.42	0.47	0.45	0.36	0.34	0.29*	0.034
Butyric acid	10.07	7.46	7.51	5.63†	5.96	8.47	6.40	4.11*	4.61*	0.013
Iso-valeric acid	2.76	2.18	2.19	1.60	1.51	2.47	1.93	1.24*	1.20*	0.014
Valeric acid	3.19	1.91	2.01	1.32†	1.21†	1.57	1.23†	0.92*	0.83*	0.024
Acetic to propionic acid ratio	2.24	2.24	2.43	2.30	2.04	1.68	1.77	2.18	2.06	0.099

*Means differ significantly with Con. diet ($P<0.05$) after Tukeys multiple comparison test, † Means differ with Con diet ($P<0.1$) after Tukeys multiple comparison test. Con. diet = mixed hay 15g DM; 10%, 20%, 30%, 40%, 60% diet = Con. diet plus freeze dried garlic leaves added at 10%, 20%., 30%, 40%, 60% DM respectively and 3%, 6%, 12%, 18% diet = Con. diet plus freeze dried garlic bulb added at 3%, 6%, 12%, 18% DM respectively.

The effect of garlic leaves or bulb in total VFA production on Rusitec is shown in figure 3.3 and individual VFA concentrations are presented in Table 3.1. The total VFA concentration (mmol/l) was significantly lower in 40% garlic leaves as well as in 12 and 18% garlic bulb group. The VFA concentration tended to be lower in 60% garlic leaves bulb and 6% garlic bulb concentration. The garlic bulb group showed the dose response effect as higher concentration resulted in reduction of total VFA concentration however this was not the case in garlic leaves group as VFA concentration lowered in 40% garlic leaves group but did not differ in 60% garlic leaves group. The reason for this inconsistency could not be understood. Busquet et al., (2005b) found that addition of garlic oil at lower concentration 3 and 30 mg/L did not affect rumen VFA concentration but at higher doses of 300 and 3000 mg/l decreased total VFA concentration in 24 h incubation but the effect was lost in dual flow continuous culture. Also, Busquet et al., (2005a) found that addition of garlic oil at 31.2 mg/L and 312 mg/L decreased ruminal VFA concentration during the first 2 days but later did not change with control diet stating that during later days rumen micro-organism might have adapted to the additives. Cardozo et al., (2004) also observed that in vitro study in dual-flow continuous culture system, the effects of plant extract on rumen microbial fermentation during the adaptation period (d 1-7) disappeared after 7 days of incubation suggesting that rumen microbes were adapted to the additives. In contrast, in the present trial, the changes in fermentation was observed and also the digestibility was decreased which suggested that the transient effect of additives were not present. Wanapat et al., (2008) observed supplementation of garlic powder had no effect on rumen VFA concentration in cattle when fed at 40, 80 and 120 g/d.

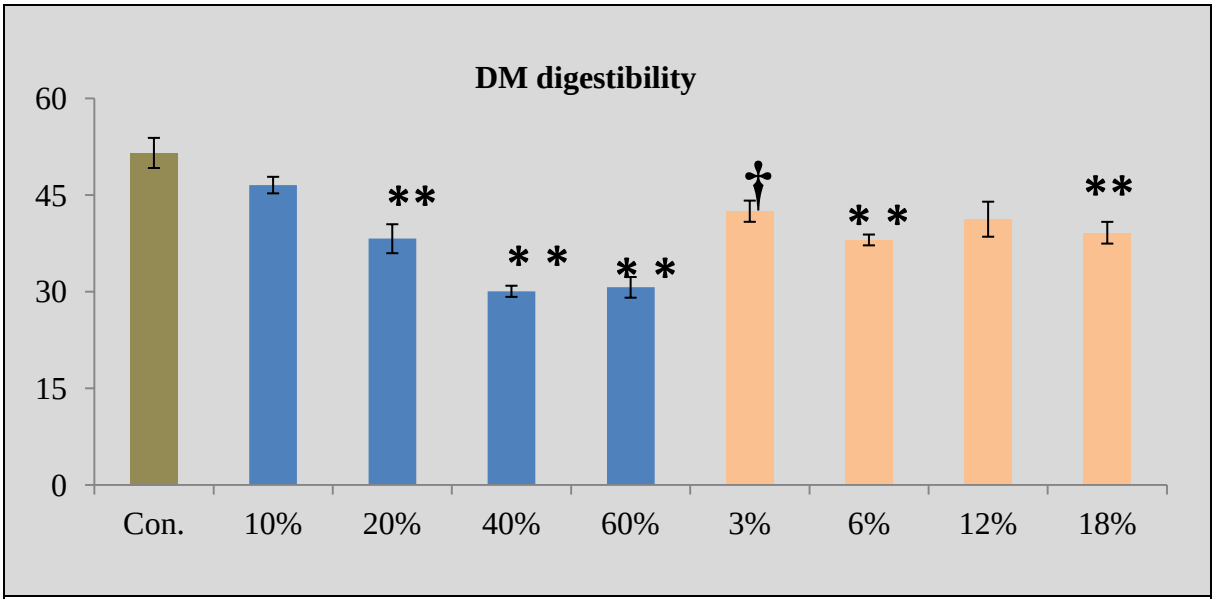


Fig 3.4 Effect of addition of garlic leaves or garlic bulb in DM digestibility on invitro fermentaion Rusitec. The values are expressed as mean ± SEM. Con= mixed hay 15g DM; 10%, 20%, 30%, 40%, 60% = Con. plus freeze dried garlic leaves added at 10%, 20%., 30%, 40%, 60% DM respectively and 3%, 6%, 12%, 18% = Con. plus freeze dried garlic bulb added at 3%, 6%, 12%, 18% DM respectively.

The effect of addition of garlic leaves or bulb in digestibility in Rusitec is shown in figure 3.4. The digestibility decreased with addition of garlic leaves or bulb at 20%, 40 % and 60% garlic leaves and in 6%, 12%, and 18% garlic bulb and tended to decrease in 3% garlic bulb group.

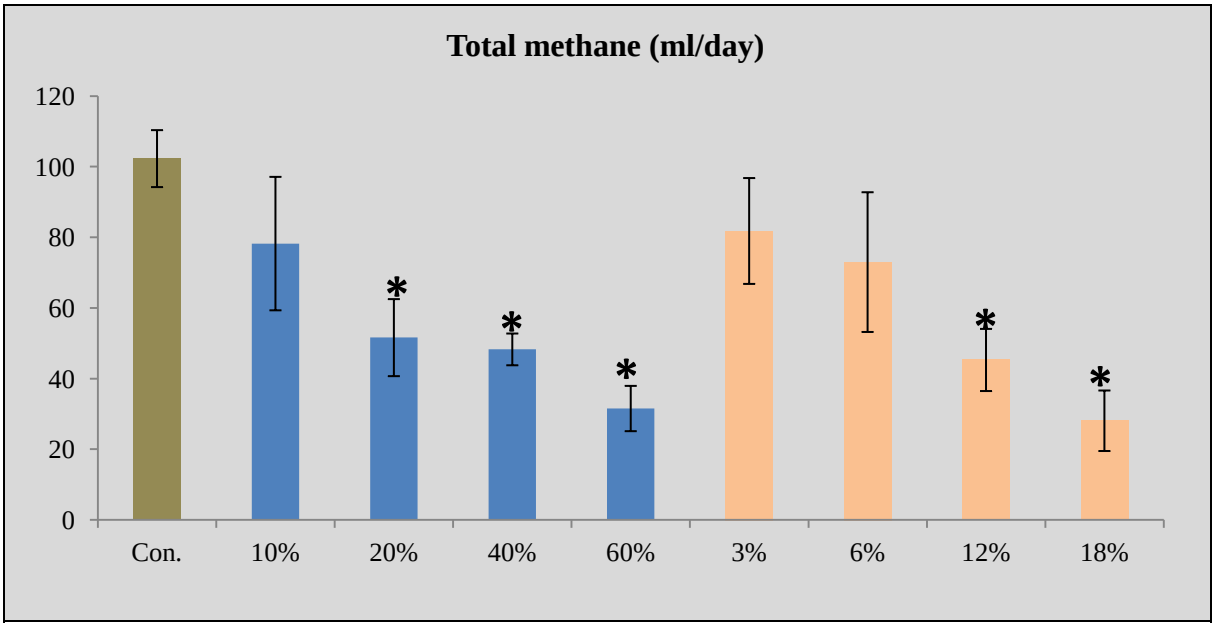


Fig 3.5 Effect of addition of garlic leaves or garlic bulb in total methane production on invitro fermentation Rusitec. The values are expressed as mean±SEM. Con.=mixed hay 15g DM10%, 20%, 40%, 60% = Con. plus freeze dried garlic leaves added at 10%, 20%., 30%, 40%, 60% DM respectively and 3%, 6%, 12%, 18% = Con. plus freeze dried garlic bulb added at 3%, 6%, 12%, 18% DM respectively.

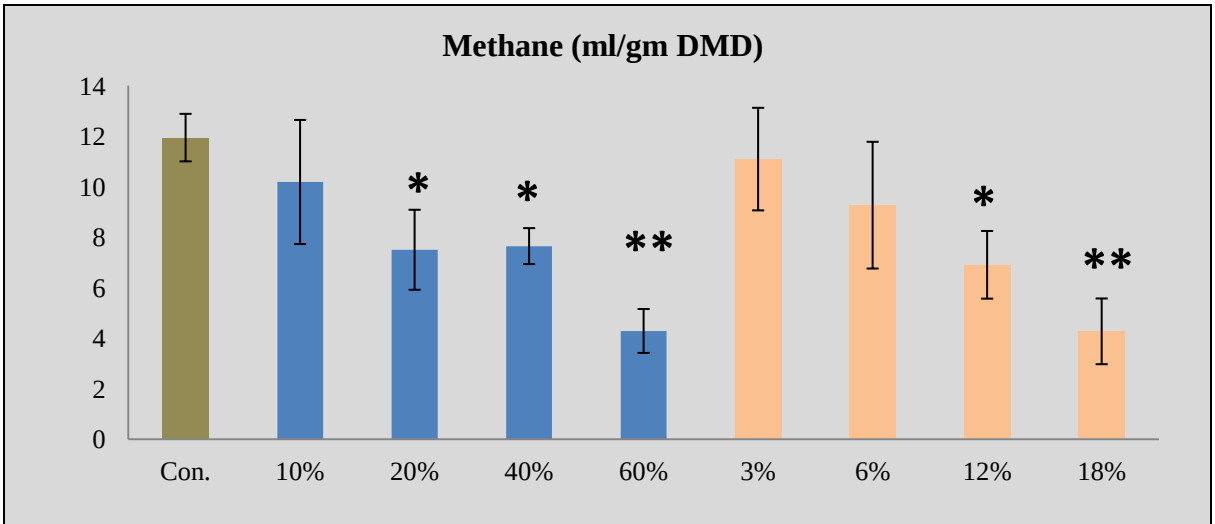


Fig 3.6 Effect of addition of garlic leaves or garlic bulb in methane emission (ml/gm DMD) on invitro fermentation Rusitec. The values are expressed as mean±SEM. Con.=mixed hay 15g DM10%, 20%, 40%, 60% = Con. plus freeze dried garlic leaves added at 10%, 20%., 30%, 40%, 60% DM respectively and 3%, 6%, 12%, 18% = Con. plus freeze dried garlic bulb added at 3%, 6%, 12%, 18% DM respectively.

The total methane emission per day and methane emission (ml/gm DMD) was significantly lower in 20%, 40% and 60% garlic leaves added diet and in 12% and 18% garlic bulb added diet. Although, the reduction in methane emission seems to be positive attribute, but there was also consequently decrease in dry matter digestibility in above mentioned groups. The reduction in methane emission with decrease in dry matter digestibility is not a good result because the aim in reduction of methane emission should not decrease digestibility of feed stuff.

Conclusion

The present experiment tested out the effect of addition of freeze dried garlic bulb and garlic bulb in invitro system using Rusitec. Addition of garlic leaves or bulb at higher concentration is not good as it decreases the total VFA concentration as well as digestibility of feedstuff with decrease in methane emission.

Chapter 4

Effect of feeding freeze dried garlic leaves on rumen fermentation, methane emission, glucose metabolism and nitrogen utilization in sheep

4.1 Introduction

Research in animal production is currently focused on developing cost effective production by improving productivity but also minimizing the impact on the environment mainly by reducing methane emission and nitrogen excretion (Kamra et al., 2012). Meanwhile, the use of chemical feed additives such as ionophores and antibiotics are discouraged due to increased concern of antibiotic resistivity and where possible natural products are encouraged in animal production (Jouany and Morgavi, 2007; Patra and Saxena, 2010). In the last decade a wide range of plants have been tested in order to reduce methane production in the rumen and some have exhibited significant effectiveness with potential practical use (García-González et al., 2008). In this regard, garlic and its bioactive components have demonstrated effects on rumen manipulation (e.g. defaunation, decreased methane production, decreased ruminal degradation of dietary proteins, reducing the proportion of acetate and increasing that of propionate) and consequently on animal production and performances (Kholif et al., 2012). Garlic is also said to influence the insulin activity in animal and thus exhibit hypoglycemic effect (Agarwal, 1996; Rivlin, 2001); but the effect of garlic on ruminant glucose metabolism are scare. Allin concentration in fresh garlic bulbs and leaves were similar in our preliminary study. Thus, based on the above characteristics of garlic, we hypothesized that freeze dried garlic leaves could have positive impact on rumen fermentation, methane emission, blood glucose kinetics and nitrogen utilization in sheep. Improved utilization of leftover garlic leaves could improve animal productivity as well as reduce the impact on environment due to less methane emission.

4.2 Materials and method

4.2.1 Animals, diet and management

Six healthy crossbred (Corriedale × Suffolk) wethers of 2 years of age weighing 46±1.2 kg of body weight were used. The experimental design, animal handling and sample collection were approved by the Animal Care Committee of Iwate University. Two dietary treatments were tested using crossover design with two 22 days periods. Control diet was mixed hay plus concentrate at 60:40 ratio. Mixed hay consisted of orchardgrass (*Dactylis glomerata*) and reed canarygrass (*Phalaris arundinacea*). The experimental freeze dried garlic leaves (FDGL) diet was prepared by adding freeze dried garlic leaves at 2.5 g/(kg $BW^{0.75}$·d) to the Control (GLCon) diet. The chemical compositions of diets are presented in Table 4.1. Sheep were offered 120 kcal/kg $BW^{0.75}$/d. Body weight measurements were taken weekly and feed was adjusted accordingly. The adaptation period to diet was 14 days in individual sheep pen and during the later 8 days sheep were moved to environmentally controlled house. The control house was maintained at 23±1°C with lighting from 8:00 h to 22:00 h with relative humidity of 70%. Feed was offered once daily at 9:00 h and fresh drinking water was available *ad libitum*. The garlic leaves were obtained from the commercial garlic farm in Aomori prefecture, Japan. Dead, dry wilted shoots and leaves were removed and only the green healthy leafy portion was used for the experiment. Garlic leaves were cut in about 2-3 cm in length and freeze dried (EYELA freeze dryer FD/5N, Tokyo Rikakikai Co., Ltd, Tokyo, Japan). The freeze dried leaves were further grinded and passed through 1mm

screen (Cyclotec ^{TM}-1903, Foss Tecator, Sweden) and fed to animal by mixing with concentrate fraction of feed.

Table 4.1 Chemical composition of diets

Chemical composition	Mixed hay*	Concentrate	FDGL
Dry matter (DM) (g/kg)	864	888	404
Crude Protein (g/kg DM)	145	152	128
Neutral detergent fiber (g/kg DM)	663	368	598
Organic matter (g/kg DM)	886	919	904
Gross energy (Kcal/g DM)	3.9	3.97	3.55

*Mixed hay: mixed hay of orchardgrass and reed canarygrass (60:40)

4.2.2 Collection of rumen fluid

Fifty mL of rumen fluid was collected on day 22 through stomach tube inserted orally before feeding (0 h), 3 h and 6 h after feeding. The pH of rumen fluid was measured as described earlier on Chapter 2. Procedures for rumen ammonia and VFA concentrations were also analyzed as described in chapter 2.

4.2.3 Methane measurement

Sheep were accustomed to open circuit respiratory chambers (50×70×100 cm) prior to the experiment. Methane gas from rumen of each sheep released from oral cavity was analyzed for 2 days continuously using methane gas analyzer (VA 3000A, Horiba Electronics, Kyoto, Japan). Span gas of 0 ppm and 159 ppm methane was used to calibrate the methane

analyzer before the experiment. 50 L/min of air inside chamber was removed continuously using the pump and a portion was fed to methane gas analyzer. Background air methane gas concentration was measured and subtracted for each sheep. Feed was offered once per day and water was available *ad libitum*. The temperature, pressure and humidity were recorded simultaneously and converted into standard temperature and pressure conditions and thus final methane volume was standardized.

4.2.4 Isotope dilution method

An isotope dilution technique using [U-^{13}C]glucose was used to measure the turnover rate of plasma glucose on day 22. Two catheters, one for isotope infusion and the other for blood sampling were inserted into left and right jugular veins on the morning of each isotope dilution technique. Catheters were filled with sterile solution of 3.8% tri-sodium citrate in order to prevent blood clotting. Three hours after feeding, 3.0 µmol/kg$^{0.75}$ of [U-^{13}C]glucose (D-glucose-$^{13}C_6$,99 atom% excess ^{13}C; Cambridge Isotope Laboratories, USA) dissolved in saline solution (0.9%) was injected as priming dose injection followed by [U-^{13}C]glucose infusion at constant rate of 3 µmol/(kg BW$^{0.75}$·h) for 4 h using multichannel peristaltic pump (AC-2120, Atto Co. Ltd. Tokyo, Japan). Blood samples were collected immediately before the priming dose and at 30 min interval during the last 2 h of the primed-continuous infusion of [U-^{13}C]glucose. The collected blood samples were transferred to heparinized tubes and stored in crushed ice until centrifugation. Blood samples were centrifuged at 8,000 × g for 10 min at 4°C and plasmas were then stored at -30°C until further analysis.

4.2.5 Nitrogen utilization

Nitrogen utilization trial was carried out for 5 days from day 15 to 19 using metabolic crates. Procedure of sample analysis has been explained previously in Chapter 2.2.7 nitrogen balance section.

4.2.6 Chemical analysis

Dry matter of feed was determined following the standard procedure of AOAC (1990). Nitrogen content in feed was determined by Kjeldahl method with the Foss Kjeltech System (Tecator Digestor System and Kjeltech 2300, Foss Tecator, Sweden). The neutral detergent fiber was determined according to Van Soest et al. (1991) using the Foss Analytical FiberCap system (FiberCap^{TM} 2021/2023, Foss Analytical, Sweden). Concentrations of rumen total VFA were determined by titrating the steam distillate of rumen fluid with 0.1 M NaOH. The titrated distillate was dried and individual VFA concentrations were determined using gas chromatography (HP 5890, Hewlett Packard, USA). The ruminal NH_3 concentration was determined using colorimetric method as described by Weatherburn (1967) using a spectrophotometer (V-630, JASCO, Japan).

Isotopic enrichments of plasma [U-^{13}C]glucose were measured as described by Fujita et al. (2006). The isotopic enrichments of plasma [U-^{13}C]glucose was determined by selected ion monitoring with gas chromatography mass spectrometry system (QP-2010, Shimadzu, Kyoto, Japan). Briefly, 1 mL of plasma was deproteinized by adding 1 mL of 4% sulfosalicylic acid (SSA). After centrifugation at 12000 × g for 10 min at 0ºC, the supernatant was purified through a tandem column containing 0.5 mL cation exchange

resin (Dowex 50W × 8, hydrogen form) and 1 mL anion exchange resin (Dowex 1 × 8, acetate form). After elution with distilled water (2 × 0.5 mL), the glucose fractions were pooled, dried and converted to pentacetate derivative with acetic anhydrate and pyrimidine. The samples were heated in the oven at 1 h at 90ºC followed by cooling on ice for 3 min. Then, 2 mL of distilled water and 1 mL of chloroform were added and mixed by vigorous shaking for 1 min (repeated thrice). The two phases were then separated by centrifugation at 3,000 × g for 5 min at 4ºC. The upper layer was discarded and the samples were then placed in glass tube and dried with nitrogen gas. 100 µL of chloroform was then added to each glass tube. The derivative was analyzed with the GC/MS with ionization using a 20eV electron beam, and the fragment was monitored at m/z 314 and m/z 319.

Plasma glucose concentration was determined enzymatically as described by Huggett and Nixon (1957). The plasma (200 µL) was deproteinized by adding 1.8 mL of trichloroacetic acid, and the liquid fraction was separated by centrifugation at 3000 × g for 10 min at 4ºC. The liquid obtained was combined with the mixed enzyme-oxygen acceptor reagents as follows: 200 mg/L glucose oxidase, 60 mg/L peroxidase and 10 mg of o-dianisidine in 10 mL of 95% ethanol. The sample was incubated at 37ºC for 1 h in a water bath and then the absorbance was measured at 440 nm using the spectrometer.

4.2.7 Calculations

The purine derivatives excretion, namely allantoin, xanthine plus hydroxanthine and uric acid from diluted urine samples were determined by the procedure described by Chen and

Gomes (1992). The amount of microbial purines absorbed (X mmol/d) corresponding to the PD excreted (Ymmol/d) was based Newton's iteration process (Chen and Gomez, 1992).

Microbial Nitrogen Supply (g N/d) =70×X (mmol/d)/0.83×0.116×1000

Where, digestibility of microbial purines was 0.83, nitrogen content of the purines was 70 mg N/mmol and ratio of purine nitrogen to total nitrogen in mixed rumen microbial biomass was 0.116.

For isotope dilution methods, the turnover rate of plasma glucose was calculated using the equation described by Tserng and Kalhan (1983) as follows:

Turnover rate = I × (1/E-1)

Where, I was the infusion rate of [U-^{13}C]glucose and E was the isotopic enrichments of [U-^{13}C]glucose during the steady state.

4.2.8 Statistical analysis

Results were presented as mean values with standard error of the mean. All data were statistically analyzed using analysis of variance with the MIXED procedure of SAS (1996). The least square means statement was used to test the effects of diet and time, with sheep as the random effect. Results were considered significant at the $P<0.05$ level, and a tendency was defined as $0.05<P<0.10$. The repeated measures statement and the Tukey's adjustment were used for time course changes and the significance level was $P<0.05$.

4.3 Result

The time course change in rumen fermentation properties is presented in Figure 4.1 and the mean values of time course change are presented in Table 4.2. Rumen pH decreased ($P < 0.01$) after feeding and was similar for 3 and 6 h after feeding in both the diets. The rumen pH did not differ between the diets ($P=0.57$) and there was no diet and time interaction ($P=0.96$). The total VFA and individual VFAs concentration increased after feeding and were similar for 3 h and 6 h after feeding. No significant differences were found in total VFA ($P=0.67$) and individual VFAs. The acetic to propionic acid ratio also did not differ ($P=0.71$) between the diets. Rumen ammonia concentration decreased ($P<0.01$) at 6 h after feeding in both GLCon diet and in FDGL diet. Rumen ammonia concentration tended to be higher ($P=0.05$) for FDGL diet.

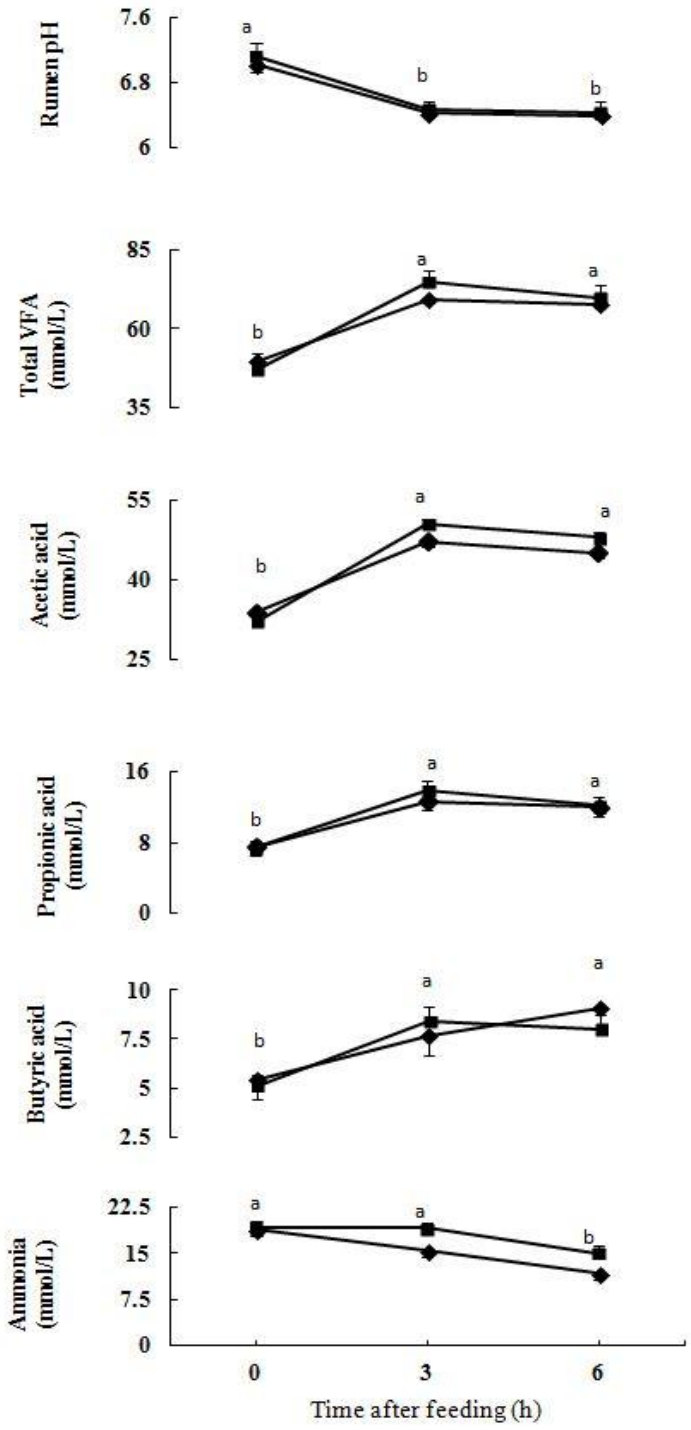


Fig 4.1 Time course change in rumen pH, total and individual VFA and rumen ammonia in sheep fed GLCon diet (♦) and FDGL diet (■). Values are expressed as mean ± SEM for n=6. Different letters (a,b) indicate significant difference between time after feeding.

Table 4.2 Dietary effects on of feeding FDGL rumen pH, concentrations of rumen ammonia and volatile fatty acids in sheep[a].

Item	Diet[b]		SEM[c]	P-value		
	GLCon	FDGL		Diet	Time	Diet*time
Rumen pH	6.61	6.67	0.100	0.574	<0.001	0.956
Total VFA (mmol/L)	62.29	63.86	3.543	0.676	<0.001	0.674
Acetic acid (mmol/L)	42.08	43.57	2.195	0.527	<0.001	0.639
Propionic acid (mmol/L)	10.76	11.25	0.772	0.551	<0.001	0.680
iso-Butyric acid (mmol/L)	0.69	0.66	0.091	0.799	0.021	0.485
Butyric acid (mmol/L)	7.39	7.16	0.610	0.713	<0.001	0.349
iso-Valeric acid (mmol/L)	0.87	0.73	0.081	0.231	<0.001	0.289
Valeric acid (mmol/L)	0.49	0.48	0.048	0.857	<0.001	0.548
Acetic/propionic acid ratio	4.02	3.97	0.134	0.706	<0.001	0.335
Rumen ammonia (mmol/L)	15.39	17.78	0.966	0.056	<0.001	0.061

[a] Values represent means of six sheep of before (0 h), 3 h and 6 h after feeding. [b] GLCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (60:40); FDGL diet: GLCon diet plus FDGL. [c]SEM, standard error of mean.

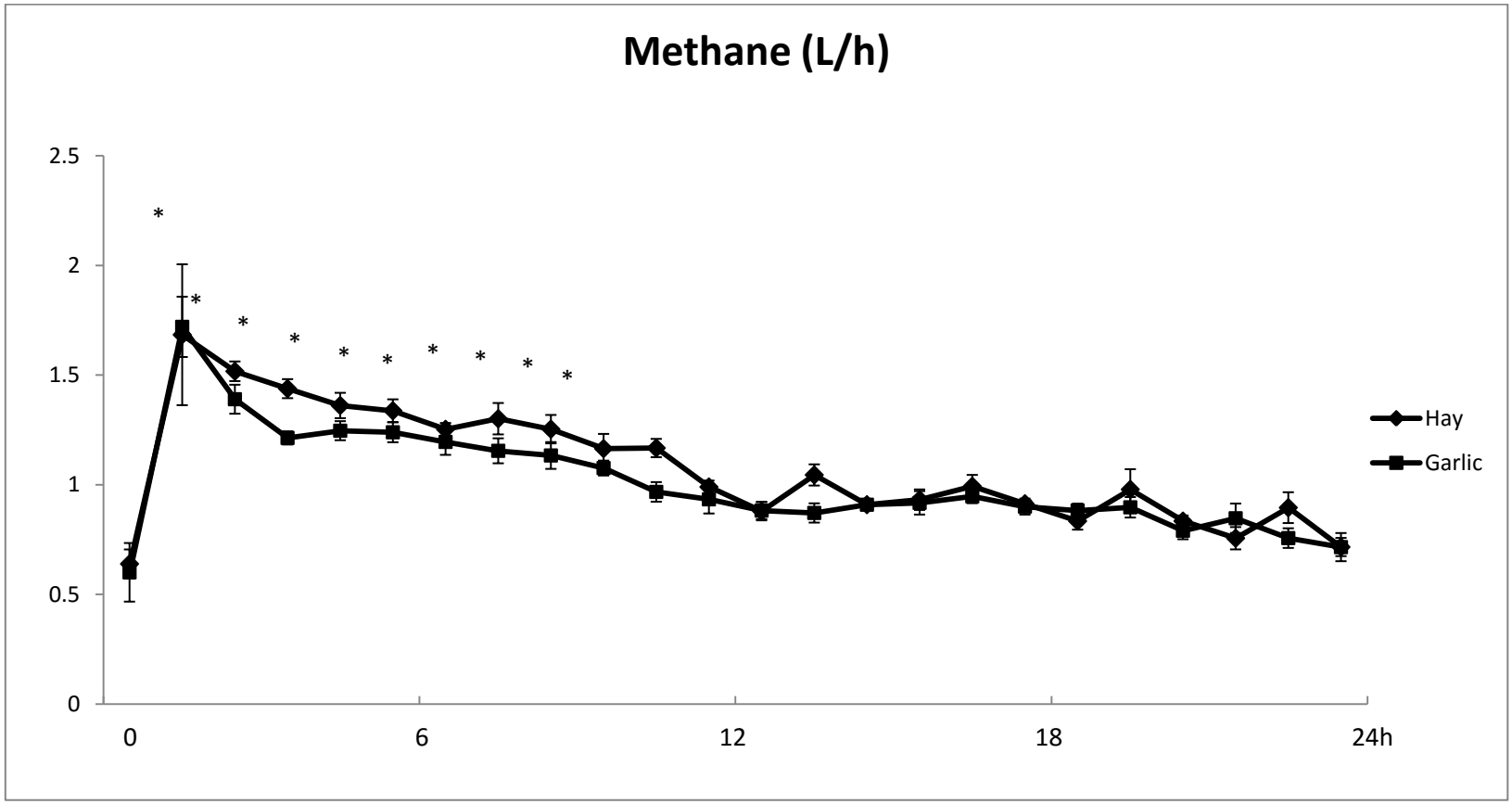


Fig 4.2 Time course changes in methane emission (L/h) in sheep fed GLCOn diet (♦) and FDGL diet (■). * indicate the difference with the methane concentration at 0 h.

Table 4.3 Effect of feeding FDGL on methane emission in sheep [a]

	Diet [b]			
Items	**GLCon**	**FDGL**	**SEM** [c]	**P-value**
Methane (L/ d)	25.79	24.38	1.124	0.279
Methane (L/(kg $BW^{0.75}$·d))	1.49	1.40	0.055	0.205
Methane (L/kg DMI)	28.05	25.34	0.919	0.042
Methane (L/kg DMD)	40.79	35.96	1.526	0.034
Dry matter digestibility (%)	68.75	70.56	0.997	0.145

[a] Values represent means of six sheep. [b] Control diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (60:40); FDGL diet: Control diet plus FDGL. [c]SEM, standard error of mean.

The methane emission per day (P=0.28) and methane emission per kg metabolic body weight per day (P=0.20) did not differ between diets. The methane emission per kg dry matter ingested (DMI) was lower (P=0.04) in FDGL diet then GLCon diet. Similarly, methane emission per kg dry matter digested (DMD) was lower (P=0.03) in FDGL diet than GLCon diet. Dry matter digestibility did not differ (P=0.14) between the diets.

Table 4.4 Effect of feeding FDGL on plasma glucose kinetics in sheep [a]

	Diet [b]			
Items	**GLCon**	**FDGL**	**SEM** [c]	**P-value**
Body weight gain (kg/d)	0.06	0.09	0.039	0.506
Basal plasma glucose concentration (mmol/L)	3.79	3.68	0.076	0.273
Plamsa glucose turnover rate (mmol/(kg $BW^{0.75}$·h))	1.39	1.59	0.083	0.092

[a] Values represent means of six sheep. [b] GLCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (60:40); FDGL diet: GLCon diet plus FDGL. [c]SEM, standard error of mean.

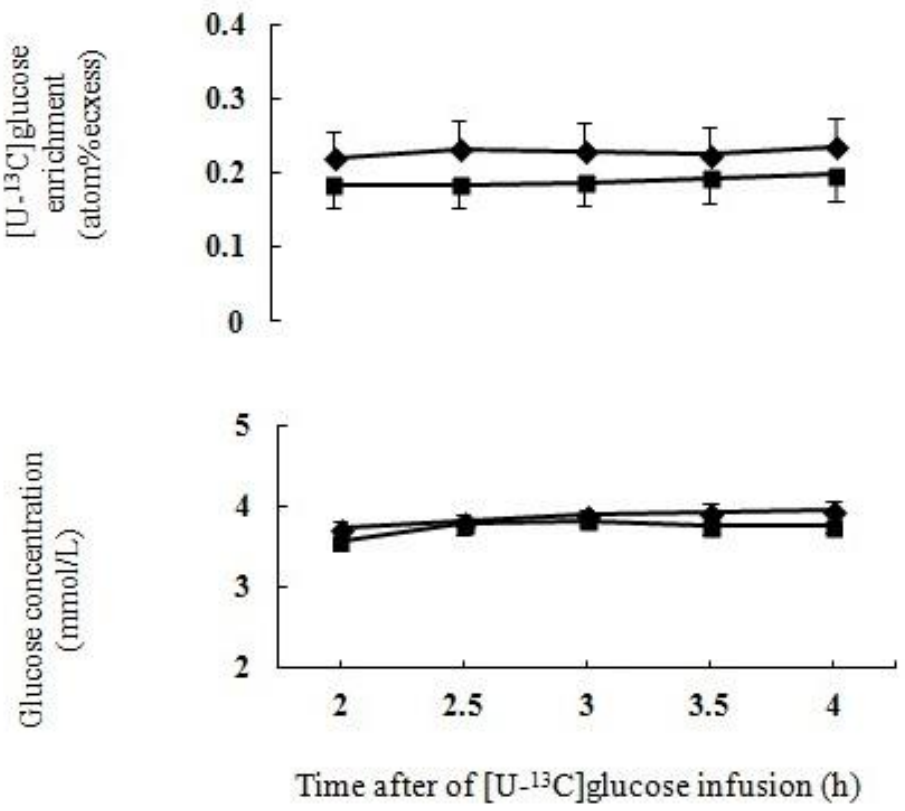


Fig 4.3 Time course change in [U-^{13}C]glucose enrichment and glucose concentration in sheep fed GLCon diet (♦) and FDGL diet (■).

Effect of feeding FDGL on plasma glucose kinetics in sheep is present in Table 4.4. No effect (P=0.51) was observed on body weight gain between two diets. Basal plasma glucose concentration did not differ (P=0.27) between the diets. Plasma glucose concentration and isotopic enrichment of [U-^{13}C]glucose were stable during the last 2 h of primed continuous infusion of [U-^{13}C]glucose in isotope dilution method (Figure 4.2) . Plasma glucose turnover rate tended to be higher (P=0.09) in FDGL diet compared to the GLCon diet.

Table 4.5 Effect of feeding FDGL on nitrogen utilization in sheep [a]

	Diet [b]			
Items	**GLCon**	**FDGL**	**SEM[c]**	**P-value**
Nitrogen intake (g/(kg $BW^{0.75}$·d))	1.19	1.25	0.001	<0.0001
Fecal nitrogen (g/(kg $BW^{0.75}$·d))	0.34	0.32	0.006	0.027
Nitrogen absorption (g/(kg $BW^{0.75}$·d))	0.85	0.92	0.006	0.001
Urinary nitrogen (g/(kg $BW^{0.75}$·d))	0.44	0.44	0.021	0.737
Nitrogen retention (g/(kg $BW^{0.75}$·d))	0.40	0.48	0.027	0.009
Nitrogen digestibility (%)	71.26	74.06	0.485	0.004

[a] Values represent means of six sheep. [b] GLCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (60:40); FDGL diet: GLCon diet plus FDGL. [c]SEM, standard error of mean.

Table 4.6 Effect of feeding FDGL on purine derivatives excretion and microbial nitrogen supply (MNS) in sheep [a]

	Diet [b]			
Items	**GLCon**	**FDGL**	**SEM[c]**	**P-value**
Allantoin (mmol/(kg $BW^{0.75}$·d))	0.37	0.43	0.029	0.084
Uric acid (mmol/(kg $BW^{0.75}$·d))	0.03	0.03	0.003	0.441
Xanthine plus hypoxanthine (mmol/(kg $BW^{0.75}$·d))	0.04	0.05	0.004	0.443
Total purine derivatives (mmol/(kg $BW^{0.75}$·d))	0.44	0.52	0.031	0.077
Total MNS (g N/(kg $BW^{0.75}$·d))	0.37	0.43	0.027	0.073

[a] Values represent means of six sheep. [b] GLCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (60:40); FDGL diet: GLCon diet plus FDGL. [c]SEM, standard error of mean.

The effect of feeding FDGL on nitrogen utilization is presented in Table 4.5 and on purine derivative excretion and microbial nitrogen supply in Table 4.6. The nitrogen intake was higher (P<0.0001) in FDGL diet. Fecal nitrogen was lower (P=0.03) in FDGL diet and thus nitrogen absorption was higher (P=0.03) in FDGL diet. Urinary nitrogen did not differ

among two diets (P=0.068) and nitrogen retention was higher (P=0.009) in FDGL diet. The nitrogen digestibility was higher (P=0.004) in FDGL diet. Allantoin excretion tended to be higher (P=0.08) in FDGL diet. Uric acid excretion (P=0.44) and xanthine plus hypoxanthine (P=0.44) did not differ among diets. As allantoin contributes major portion of total purine derivatives excretion, the total purine derivatives excretion in FDGL diet tended to be higher (P=0.08) in FDGL diet. Based on total purine derivative excretion calculations the total microbial nitrogen supply in FDGL diet also tended to be higher (P=0.07) in FDGL diet.

4.4 Discussion

4.4.1 Rumen fermentation characteristics

As observed in our experiment, the average pH was above 6 for both diets which indicate that the rumen environment was favorable for normal fermentation process (Dijkstra et al., 2012). The results are consistent with other studies (Yang et al., 2007; Hodjatpanah et al., 2010; Kamruzzaman et al., 2011) where rumen pH values did not differ on addition of garlic oils, garlic components or garlic leaf silage respectively. The observed lower concentration of rumen pH after feeding can be linked to higher VFA concentration in the rumen, as the negative relationship between VFA concentration and pH in rumen fluid (Dijkstra et al., 2012).

The volatile fatty acid pattern reflects the efficiency of nutrient utilization, acetate and butyrate synthesizing pathways are hydrogen producers and propionate pathway is hydrogen consumer and therefore an increase in propionate production is generally

associated with a decrease in methane emission. Patra et al. (2007) fed garlic bulb at the rate of 1% dry matter intake to buffalo, Chaves et al. (2008) fed garlic oil at 0.2 g/kg dry matter to lambs and Wanapat et al. (2008) fed graded levels of garlic powder (80-120 g/d) along with treated urea rice straw to steers and did not find any changes in rumen total VFA concentrations. Similar lack of effect was also found by Busquet et al. (2005), Klevenhusen et al. (2011) and Garcia-Gonzalez et al. (2008) in *in-vitro* experiments. These findings suggest that inclusion of garlic or its component in diet did not have negative effect on carbohydrate fermentation and similar effects can be observed when fed with freeze dried garlic leaves.

Ruminal ammonia nitrogen has been reported to be an important nutrient as this is considered to be the major source of nitrogen for microbial protein synthesis. Wanapat and Pimpa (1999) observed higher level of rumen ammonia nitrogen was associated with higher nutrient digestibility and intake. Zhu et al. (2012) found an increase in ruminal ammonia when infused garlic oil in rumen at 0.8g/d in goats. Yang et al. (2007) reported that garlic diet increased ruminal ammonia concentration in growing lambs and lactating cows, when garlic fed at 5g/d. The present experimental findings did not support the findings reported previously (Cardozo et al., 2004; Klevenhusen et al., 2011; Tao et al., 2016) who supplemented various garlic components *in-vitro* and in sheep diets and reported decrease in ammonia concentration. Wanapat et al. (2008) showed that addition of garlic powder did not influence the rumen ammonia concentration in steers. Exact mechanism for these unpredictability are yet clearly not understood, however difference in feeding pattern and

diet might be responsible for this dissimilarity, as animals in the present experiment were offered freeze dried garlic leaves supplemented to the diet.

4.4.2 Methane emission

Patra et al. (2011) fed raw garlic at 1% dry matter intake and found reduction of 11% methane emission in sheep fed concentrate-roughage diet at 1:1 ratio in open circuit respiratory chamber. Klevenhusen et al. (2011) failed to observe similar reduction in methane emission when fed 5 g garlic oil, but 2 g diallyldisulfide/kg dry matter in sheep tended to decrease methane production per kg organic matter digested. In our study FDGL diet reduced methane per kg dry matter intake was well as per kg dry matter digested without reduction in dry matter digestibility, suggesting that garlic improves digestibility and energy use efficiency. The antimicrobial activity of garlic has been attributed to the presence of organosulfur compounds, and particularly to the allicin which might have caused change in microbial population in rumen causing the reduction in methane emission. Some authors suggest that the sulfhydryl groups of the organosulfur compounds found in garlic are the main active site, and modify the microbial metabolism through their interaction with the other sulfhydryl groups of microbial protein and biological active molecule by a thioldisulfide exchange reaction (Reuter, 1995). The capacity of sulfhydryl containing enzymes participating in different activities of *archea* (methanogens) metabolism (Gebhardt and Beck, 1996) decreases methane emission. Miller and Wolin (2001) demonstrated by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase by sulfhydryl compounds, it has the potential to specifically inhibit rumen methanogenic

archea without affecting rumen bacteria due to their different membrane lipid composition. Thus, reduction in methane without reduction in total VFA might be due to of selective inhibition of methanogens in the current study by FDGL supplement. The mode of action of garlic compounds with respect to their anti-methanogenic effects have not yet been identified in detail. Although direct effects against methanogens are probable, indirect effects are also possible through suppression of ruminal protozoa or ruminal fiber degradation or both or lower supply of hydrogen to the methanogens (Martin et al., 2010).

4.4.3 Glucose metabolism

Plasma glucose concentration was similar between the diets which is in accordance with Chaves et al. (2008) and Anassori et al. (2015) where plasma glucose concentration did not differ when garlic was fed to growing stage or mature sheep respectively. Garlic has long been claimed to possess a hypoglycemic effect (Agarwal, 1996), attributed due to an increase in serum insulin level (Anassori et al., 2015). However, our result contrasts with Kamruzzaman et al. (2011), Kholif et al. (2012) and Pirmohammadi et al. (2014) where feeding garlic silage or garlic components increased basal plasma glucose levels. They suggested the increase might be associated with higher propionate concentration in rumen and propionate is the main precursor of glucose in ruminants. In our study, we did not find difference in propionate concentration among the diets which may thus have not been reflected in the plasma glucose level.

To our knowledge no information is reported on the effects of garlic leaves on glucose turnover rate in sheep. The determination of glucose turnover gives more dynamic

information about situation of the substrate *in-vivo*, rather than the determination of glucose concentration only in plasma. It helps us to determine the glucose recycling in the body i.e. glucose disposal and hepatic glucose production while in a steady state and is measured by using stable isotope using isotope dilution technique. The glucose turnover rate tended to be higher in FDGL diet in our study which might be because of enhancement of glucose utilization by increasing the pancreatic secretion of insulin from existing β-cells as reported by Eidi et al. (2006) in rats. Similarly, increased insulin concentration increased glucose turnover rate (Cherrington and Vranic, 1974) in both normal and pancreatic dogs. Sano et al. (1999) also found that glucose turnover rate increased with increased concentration of insulin in sheep. S-Allyl cysteine sulfoxide and diallyl trisulfide, two major active sulfur compounds of garlic, were shown to have potent insulin secretagogue activity (Augusti and Sheela, 1996; Liu et al., 2005). In the present experiment, though insulin concentration was not measured, howeverwe speculate that the active sulfur compounds in garlic might be involved in higher insulin production and thus influencing the turnover rates of glucose.

4.4.4 Nitrogen utilization

Studies on plant secondary metabolites on ruminants have focused their potential to improve nitrogen utilization as it serves both to solve problem of productivity and environment. Nitrogen retention is the index of protein status in ruminants and microbial protein reaching the duodenum represents the greatest contribution of protein in ruminants. Microbial protein has relatively good amino acid balance, thus it is necessary that it should be maximized for efficient use of feed protein and energy (Chen and Gomez, 1992). In our

experiment, the nitrogen intake in the FDGL diet was higher because of extra nitrogen added by garlic leaves supplement. Fecal nitrogen was lower in FDGL diet which is because of higher digestibility of diet. Urinary nitrogen was similar between both diets. Nitrogen absorption and nitrogen retention is higher in FDGL diets. Previous studies on nitrogen balance in sheep have shown that garlic or its constituents have shown positive effects. Kamruzzaman et al. (2011) fed 10% of hay replaced by garlic leaves and found increased nitrogen absorption, nitrogen digestibility and microbial nitrogen supply in sheep. Tao et al. (2016) also found that supplementing 2 g allin/head/d improved fecal nitrogen, nitrogen retention and nitrogen digestibility in sheep. Wanapat et al. (2008) supplemented garlic powder at 40, 80 and 120 g/d did not affect fecal nitrogen, urinary nitrogen was decreased while nitrogen retention was increased only at 120 g/d in steers. However, the exact mechanism for positive nitrogen balance is yet not clear. According to Amagase et al. (2001) and Kamruzzaman et al. (2011) garlic could act as remedy for intestinal disorder, flatulence, worms and respiratory infections. Such positive effect in digestive tract might be one of the reasons for increased digestibility and increased nitrogen balance.

4.5 Conclusion

Dietary supplementation of garlic leaves decreased methane emission and increased nitrogen utilization without any adverse effect on rumen fermentation. Positive impact on glucose turnover could improve efficacy of feeding which in turn might improve production performance in sheep. Although, the exact cause is yet unclear, plant secondary compounds present in garlic leaves may have several independent actions or mixed effect

and their relation to each other in their biochemical consequences. Also growing conditions may significantly affect the chemical composition of garlic and thus the concentration of bio-active compounds (Martins et al., 2016), caution must be taken to generalize the results. However, there is a great potential in garlic to be used as a feed additive for economic and eco-friendly livestock production.

Chapter 5

Effect of feeding garlic oil on rumen fermentation, methane emission, nitrogen utilization, and plasma glucose and amino acid kinetics in sheep

5.1 Introduction

Previous studies were carried out using garlic leaves and garlic bulb in invitro system and also using freeze dried garlic leaves in invivo system using garlic leaves. Based on the previous findings, we tried to see the effects of garlic oil on rumen fermentation, methane emission, nitrogen utilization and plasma glucose and amino acid concentration as garlic oil has more concentration of active ingredients compared to leaves and bulb, thus the effects may be more pronounced using garlic oil. Earlier studies using garlic oil by (Busquet et al., 2005b; Chiang et al., 2006; Kholif et al., 2012) were on rumen fermentation characteristics, methane emission, and/or nitrogen balance in ruminants, however through this experiment we tried out to further explore the study by using isotopic studies on glucose, plasma amino acid kinetics and whole body protein synthesis also. Furthermore, we also tried through the experiment to the activity of garlic oil on glucose metabolism using the hyperinslunemic euglycemic clamp.

5.2 Materials and methods

5.2.1 Animal diets and management

The experimental design, animal handling and sample collection were approved by Animal Care Committee of Iwate University. Six healthy wethers (Corriedale × Suffolk crossbred, 49.2 ± 2 kg of initial BW) were used in the experiment. The experiment was conducted using a crossover design with 22 days periods, each consisting of 14 days of adaptation and 8 days of sampling. Two dietary treatments one of mixed hay and concentrate (GOCon) diet and garlic oil added to control diet (GO) diet was used during the experiment. The

GOCon diet consisted of mixed hay (Orchardgrass and Reed canarygrass) and commercial concentrate at ratio of 70:30. The GO diet consisted of garlic oil added to GOCon diet at rate of 30 mg/kg BW $^{0.75}$/d.

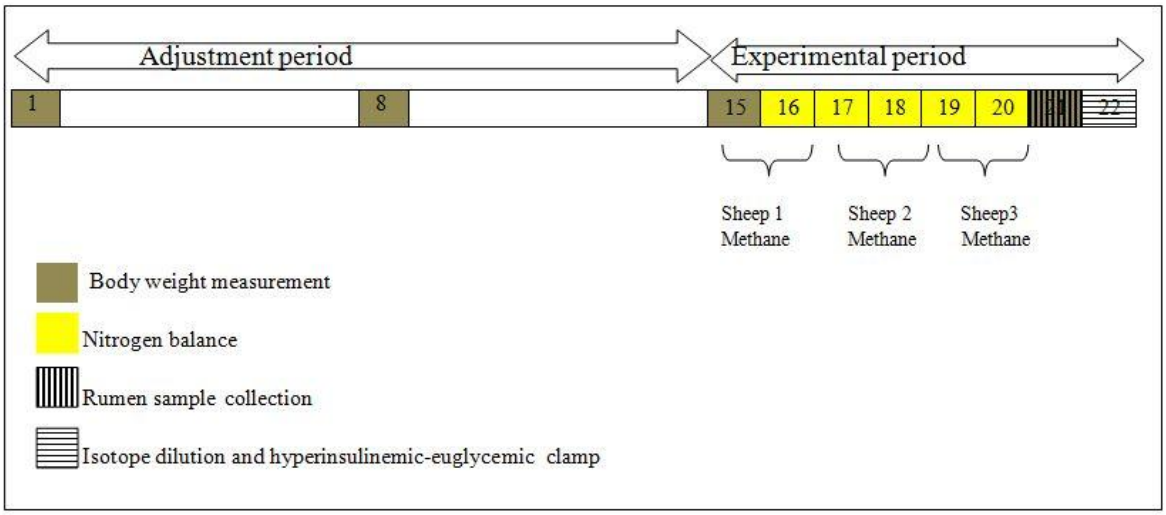


Fig 5.1 Experimental protocol showing experimental design

5.2.2 Sample collection

All procedures of chemical analysis of feed and the sampling procedure and treatments of urine, feces and ruminal fluid have been described in detail in Chapter 2.

5.2.3 Methane gas emission measurement

The procedures for methane gas emission measurement has already been explained in Chapter 4.

5.2.3 Isotope dilution and hyperinsulinemic-euglycemic clamp method

On day 22 of each dietary treatment, an isotope dilution of [U-^{13}C]glucose, [1-^{13}C]leucine, [$^{2}H_5$]Phenylalanine and [$^{2}H_2$]Tyrosine was conducted to determine the kinetics of plasma glucose, leucine, phenylalanine and tyrosine in sheep. Experimental layouts are shown in Figure 5.1 and Figure 5.2. Two catheters, one for isotope dilution and another for blood sampling, were inserted into the both jugular veins on the morning of isotope dilution method. Catheters were filled with a sterile solution of 0.13 mol/L trisodium citrate. In brief, on the day of isotope dilution method, saline solution containing 7.2 µmol/L $BW^{0.75}$ of [1-^{13}C]Leucine (L-leucine-1-^{13}C, 99 atom % excess ^{13}C; Cambridge Isotope Laboratories, USA), 2.9 µmol/L $BW^{0.75}$ of [U-13C]glucose (D-glucose-$^{13}C_6$, 99 atom % excess ^{13}C; Cambridge Isotope Laboratories, USA), 3.0 µmol/L $BW^{0.75}$ of [$^{2}H_5$]Phenylalanine (L-phenylalanine, ring-D_5, 98%; Cambridge Isotope Laboratories, Inc., USA), 1.8 µmol/L $BW^{0.75}$ of [$^{2}H_2$]Tyrosine (L-4-hydroxyphenyl-2,3,,5,6-D_4-alanine, 98 atom%; Isotec Inc., USA) and 1.8 µmol/L $BW^{0.75}$ of [$^{2}H_2$]Tyrosine (L-tyrosine, ring 3.5-D_2, 98%; Cambridge Isotope Laboratories Inc., USA) was injected into the jugular infusion catheter as a priming dose. The tracer solution was continuously infused over four hours period immediately after priming injection using a multichannel peristaltic pump (AC-2120, Atto, Japan) at a rate of 7.2 µmol/L $BW^{0.75}$/h [1-13C]leucine , 2.9 µmol/L $BW^{0.75}$/h [U-^{13}C]glucose, 3.0 µmol/L $BW^{0.75}$/h, [$^{2}H_5$]phenylalanine and 1.8 µmol/L $BW^{0.75}$/h . Blood samples were taken immediately before (10 mL) and 30 minutes interval (5 mL) over the last 2 h of isotope infusion. The blood samples were transferred to heparinized tubes and stored

temporarily in crushed ice. Blood samples were centrifuged at 8000 × g for 10 min at 4ºC and plasma were harvested from blood cells and was stored at -30ºC for later analysis.

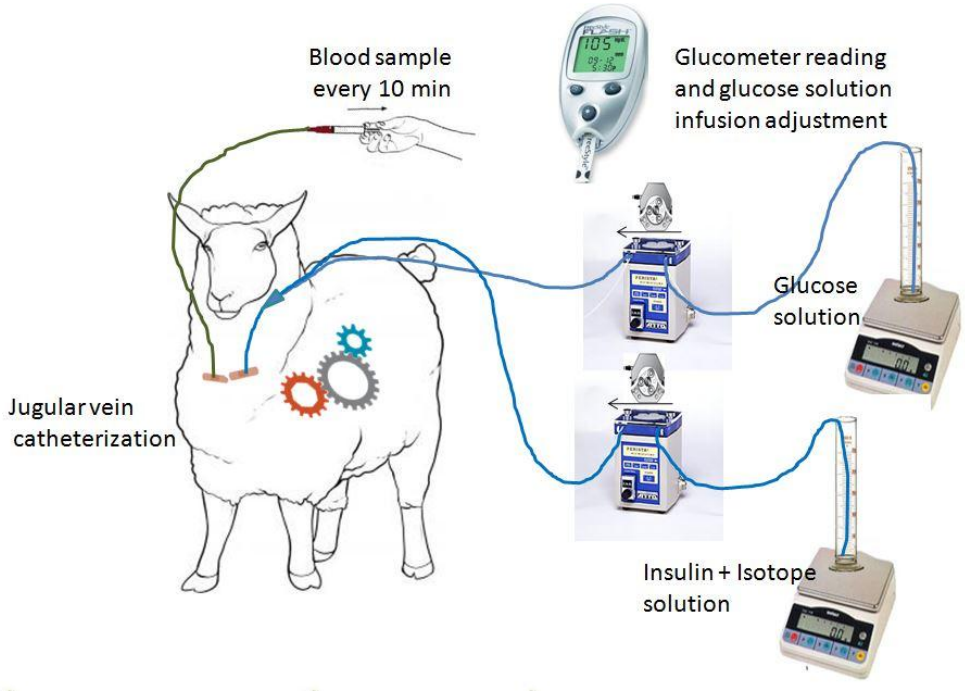


Fig 5.2 Figure illustrating hyperinsulinemic-euglycemic clamp

On day 22 of each dietary treatment, the hyperinsulinemic euglycemic clamp was carried out during the last two hours period of isotope dilution method. Sterile saline (0.9% sodium chloride) solutions of insulin 100 units/ml (Novolin R, Novo Nordisk, Denmark) was continuously infused through peristaltic pump at rate of 6 mU/kgBW/min. During the insulin infusion, 2 ml of blood was taken every 10 minutes and blood glucose concentration was measured using glucometer (Freedom Freestyle Lite, Abbott Laboratories, Abbott Park, IL, USA). A glucose solution (20% W/V was variably infused through the same catheter

via the other peristaltic pump based on glucometer reading to maintain the euglycemic blood glucose level.

5.2.5 Chemical analysis

The enrichments of plasma [U-^{13}C]Glucose, [1-^{13}C]Leucine, α-[1-^{13}C]KIC, [$^{2}H_5$]Phenylalanine , [$^{2}H_4$]Tyrosine and [$^{2}H_2$]Tyrosine were determined using a gas chromatography mass spectrometry (QP-2010, Shimadzu Corp., Japan) with selected ion monitoring. The following ions were monitored: m/z 302 and 303 for leucine, m/z 302 for n-leucine, m/z 245 for ketovaleric acid (KVA), m/z 259 and 260 for α-keto isocaproic acid. (α-KIC). The measurements were carried out according to procedure of Rocchicciolo et al., (1981) and Calder and Smith (1998). Briefly, 1 mL of plasma was deproteinized by adding 1 mL of sulfosalicylic acid (SSA) and the internal standard, 100 µL of n-Leu (0.5 mmol/L) and 100 µL of KVA (0.05 mmol/L). After centrifugation at 12000 × g for 10 min at 0ºC, the supernatant was applied to a column containing 0.5 mL of cation exchange resin (Dowex 50W × 8, hydrogen form) and the column was washed with distilled water (2 × 0.5 mL). The eluent obtained was submitted to α-KIC analysis. Subsequently, 4 N NH_4OH was passed through the column (2 × 1 mL) and washed again with distilled water (1 ml). A 0.5 mL aliquot of the resulting eluent was collected in a screw-capped glass tube and dried in desiccators containing anhydrous $CaCl_2$ and H_2SO_4 for leucine analysis. For the α-KIC fraction, 1 mL of eluent was mixed with 0.5 mL of 1% *o*-phenylediamine in 4N HCl solution in a screw-capped glass tube and heated for 1 h at 90ºC followed by cooling for 1 h at room temperature. Then, 2 mL of ethyl acetate was mixed by vigorous shaking for 1 min

and centrifuged at 1000 × g for 10 min at 4ºC. The supernatant was separated and dried using anhydrous Na_2SO_4 for 2 h, and the supernatant was then placed in a new screw-capped glass tube and dried with nitrogen gas. After all of the samples (for leucine and α-KIC analysis) were dried, 25 µL of acetonitrile and 25 µL of *N*-methyl-*N*-t-butyl-dimethylsilyltrifluroacetamide (MTBSTFA) were added to each screw-capped glass tube. The tubes were capped and then heated at 80ºC for 20 min. Two samples were then mixed in chromatographic vials and analyzed in GC/MS.

The plasma [U-^{13}C] glucose enrichment (atom % excess) was measured as described by Tserng and Kalhan (1983), with slight modification described by Fujita et al., (2006) as described earlier in Chapter 4. The concentrations of plasma phenylalanine and tyrosine and enrichments of plasma [$^2H_5$]phenylalanine, [$^2H_4$]tyrosine and [$^2H_2$] tyrosine were determined using a GC/MS (QP-2010, Shimadzu Corp., Japan) by procedures of Rocchicciolo et al. (1981) and Calder and Smith (1998) as described by Fujita et al. (2006). Briefly, 1 mL of blood plasma was deproteinized by adding 1 mL of 4% SSA, and the internal standard 100 µL of α-methyl-phenylalanine (mPhe, 0.5 mmol/L) and 100 µL of α-methyl-tyrosine (mTyr, 0.5 mmol/L) and kept in refrigerator for 30 min. After centrifugation at 12000 × g for 10 min at 0ºC, the supernatant was applied to a column containing 0.5 mL of cation exchange resin (Dowex 50W × 8, hydrogen form) and the column was washed with distilled water (2 × 0.5 mL). Subsequently, 1 mL of 4 N NH_4OH was added twice and washed finally with 1 mL distilled water. A 0.5 mL of eluent was collected in screw-capped tube and dried in desiccators. After drying, samples were mixed with 25 µL of acetinitrile and MTBSTFA, heated at 80ºC for 20 min. Enrichments were

then measured by GC/MS. The ions monitored are m/z 336 for phenylalanine, 341 for [$^2H_5$]phenylalanine, 466 for tyrosine, 468 for [$^2H_2$]tyrosine and 470 for [$^2H_4$]tyrosine.

5.2.6 Calculations for Rate of production (Rp) and Rate of utilization (Ru) of glucose

The rate of production and utilization of glucose during the last 120 min of [U-13C]glucose isotope dilution was determined using non-steady state equation (Cowan and Hetenyi, 1971) as described by (Sano et al., 1996).

$$Rp = (I-p\cdot V\cdot ((C_1+C_2)/2\cdot ((E_2-E_1)/ (T_2-T_1)))\cdot(1/E_2+E_1)\cdot 2-1)$$

$$Ru = Rp - p\cdot V\cdot ((C_2-C_1)/(T_2-T_1)$$

Where, I is the infusion rate of [U-13C]glucose, C_1 and C_2 are the plasma glucose concentrations (mg/mL), and E_1 and E_2 are the enrichments of plasma [U-13C]glucose (mol% excess) at T_1 and T_2 (min) respectively. V is the volume of glucose and p is the pool fraction. The distribution volume was assumed to be 179 mL/kg BW (Weekes et al., 1983) and the pool fraction was assumed to be 0.65 (Brockman, 1979).

5.3 Results

The time course change in rumen fermentation properties is presented in figure 5.3 and the mean values of time course change are presented in Table 5.1. Rumen pH decreased ($P<0.01$) after feeding and was similar for 3 h and 6 h after feeding in both the diets. The rumen pH did not differ between the diets ($P=0.689$) and there was no diet and time interaction ($P=0.787$).

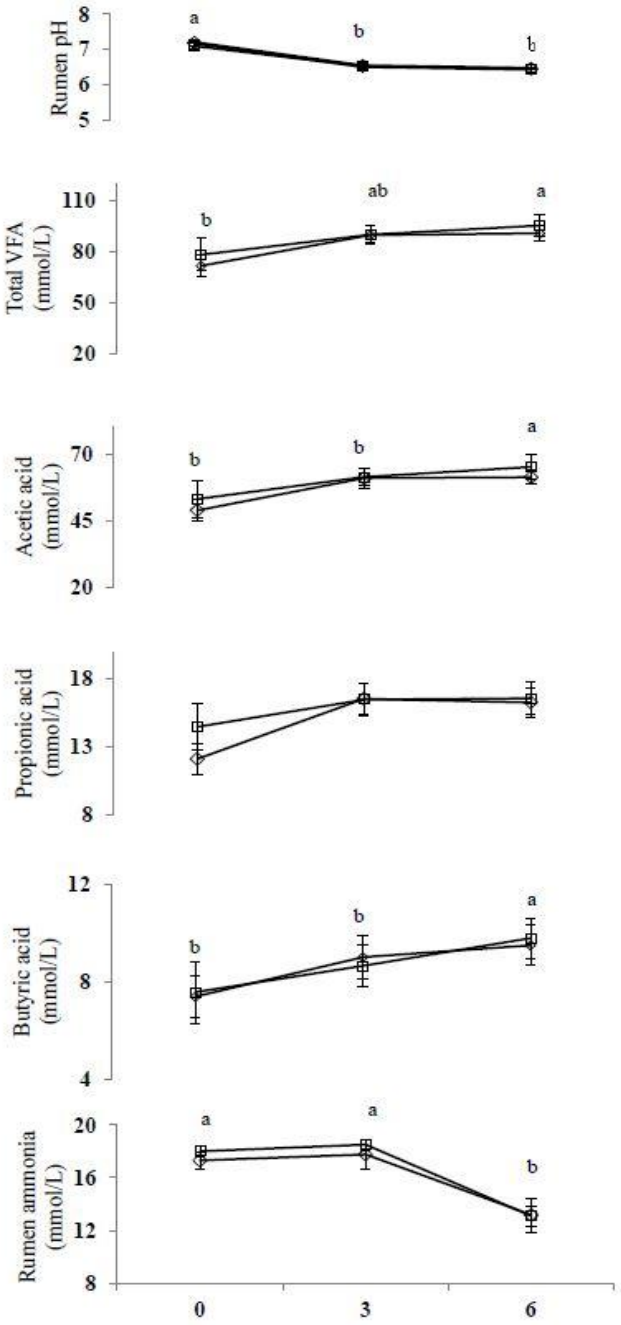


Fig 5.3 Time course change in rumen pH, total and individual VFA and rumen ammonia in sheep fed GOCon diet(- ◊-)and garlic oil diet(-□-). Values are expressed as mean ± SEM for n=6. Different letters (a,b) indicate significant differences ($P<0.05$) between time after feeding.

The total VFA concentrations increased after feeding at 6h and total VFA concentration did not differ with 0h and 6h indicating at 3h indicating it was at intermediate stage of VFA concentration rise. No significant differences were found in total VFA (P=0.506) and individual VFAs. The acetic to propionic acid ratio also did not differ (P=0.758) between the diets. Rumen ammonia concentration also did not differ between the diets (P=0.69).

Table 5.1 Dietary effect of feeding garlic oil on rumen pH, concentrations of rumen ammonia and volatile fatty acids in sheep [a].

Item	Diet[b]		SEM[c]	P-value		
	GOCon	Garlic Oil		Diet	Time	Diet*time
Rumen pH	6.72	6.69	0.051	0.689	<0.0001	0.787
Total VFA (mmol/L)	83.84	87.67	3.816	0.506	0.037	0.873
Acetic acid (mmol/L)	56.95	59.78	2.565	0.470	0.044	0.893
Propionic acid (mmol/L)	14.98	15.85	0.729	0.438	0.046	0.594
iso-Butyric acid (mmol/L)	1.00	1.11	0.111	0.472	0.199	0.513
Butyric acid (mmol/L)	8.63	8.67	0.675	0.962	0.056	0.880
iso-Valeric acid (mmol/L)	1.21	1.46	0.223	0.333	0.153	0.228
Valeric acid (mmol/L)	1.07	0.81	0.139	0.183	0.813	0.566
Acetic/propionic acid ratio	3.85	3.79	0.125	0.758	0.721	0.458
Rumen ammonia (mmol/L)	16.09	16.51	0.769	0.691	<0.0001	0.553

[a] Values represent means of six sheep. [b] GOCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (70:30); Garlic Oil diet: GOCon diet plus garlic oil @30 mg/kg $BW^{0.75}$/d. [c] SEM, standard error of mean.

Table 5.2 Effect of feeding garlic oil on methane emission in sheep [a]

Items	Diet [b]		SEM [c]	P-value
	GOCon	Garlic Oil		
Methane (L/ d)	25.04	25.43	0.579	0.539
Methane (L/(kg $BW^{0.75}$·d))	1.31	1.33	0.034	0.660
Methane (L/kg DMD)	32.17	32.34	1.073	0.886
Dry matter digestibility (%)	68.75	70.56	0.997	0.145

[a] Values represent means of six sheep. [b] GOCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (70:30); Garlic Oil diet: GOCon diet plus garlic oil @30 mg/kg $BW^{0.75}$/d. [c]SEM, standard error of mean.

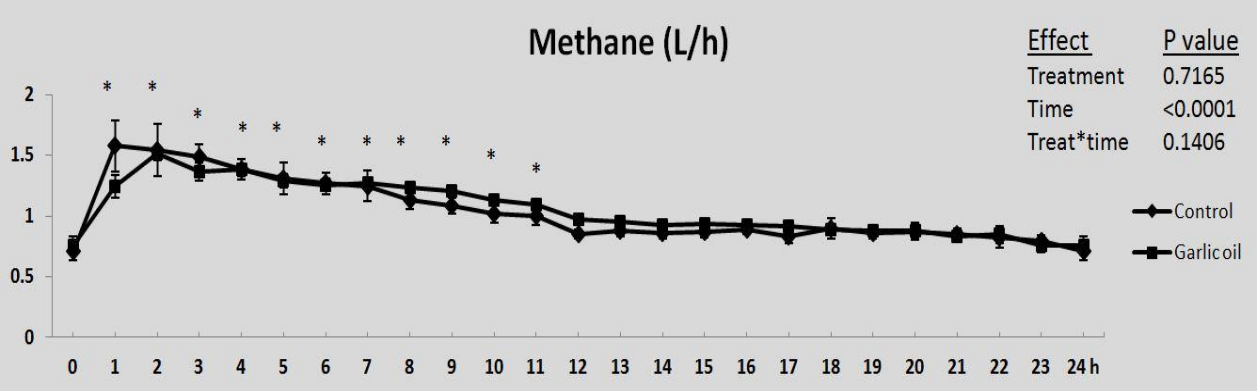


Fig 5.4 Time course changes in methane emission (L/h) in sheep fed Control (GOCon) diet (♦) and Garlic Oil diet (■).

The effect of feeding garlic oil on methane emission in sheep is presented in Table 5.2 and methane emission per hour in 24 h period is presented in Fig 5.4. The methane emission (P=0.54) did not differ between the diets and similarly methane emission per kg metabolic body weight per day (P=0.66) did not differ between diets. The methane emission per kg dry matter digested also did not differ between the diets (P=0.886) and dry matter digestibility also did not differ between the diets (P=0.145).

Table 5.3 Effect of feeding garlic oil on nitrogen utilization in sheep [a]

	Diet [b]			
Items	**GOCon**	**Garlic Oil**	**SEM[c]**	**P-value**
Nitrogen intake (g/(kg $BW^{0.75}$·d))	1.24	1.24	-	-
Fecal nitrogen (g/(kg $BW^{0.75}$·d))	0.43	0.38	0.006	0.004
Nitrogen absorption (g/(kg $BW^{0.75}$·d))	0.81	0.86	0.008	0.004
Urinary nitrogen (g/(kg $BW^{0.75}$·d))	0.54	0.50	0.037	0.425
Nitrogen retention (g/(kg $BW^{0.75}$·d))	0.28	0.36	0.038	0.120
Nitrogen digestibility (%)	65.59	69.67	0.643	0.004

[a] Values represent means of six sheep. [b] GOCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (70:30); Garlic Oil diet: GOCon diet plus garlic oil @30 mg/kg $BW^{0.75}$/d. [c]SEM, standard error of mean.

The effect of feeding garlic oil on nitrogen utilization is presented in Table 5.3. The nitrogen intake is similar between the diets as there was no feed refusal due to administration of garlic oil in animal. Fecal nitrogen was lower (P=0.004) in garlic oil diet as a result N absorption was higher (P=0.004) in garlic oil diet. Urinary nitrogen did not differ (P=0.425) between the diets thus N retention also did not differ (P=0.120) between the diets. N digestibility was higher (P=0.004) in garlic oil diet.

Table 5.4 Effect of feeding garlic oil on plasma glucose kinetics in sheep [a]

Items	Diet [b]		SEM [c]	P-value
	GOCon	Garlic Oil		
Basal plasma glucose concentration (mmol/L)	3.28	2.99	0.118	0.039
Plamsa glucose turnover rate (mmol/(kg $BW^{0.75}$·h))	1.12	1.32	0.030	0.002

[a] Values represent means of six sheep. [b] GOCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (70:30); Garlic Oil diet: GOCon diet plus garlic oil @30 mg/kg $BW^{0.75}$/d. [c]SEM, standard error of mean.

The effect of feeding garlic oil on plasma glucose kinetics is presented in Table 5.4. The time course change in plasma glucose concentration and plasma [U-^{13}C]glucose enrichments during the isotope dilution is shown in Fig 5.5. Plasma glucose concentration was lower (P=-.039) in garlic oil diet. The isotopic enrichments of plasma [U-^{13}C]glucose were steady during the isotope dilution process as shown in fig 5.5. Plasma glucose turnover rate was higher (P=0.002) in garlic oil diet compared to control diet.

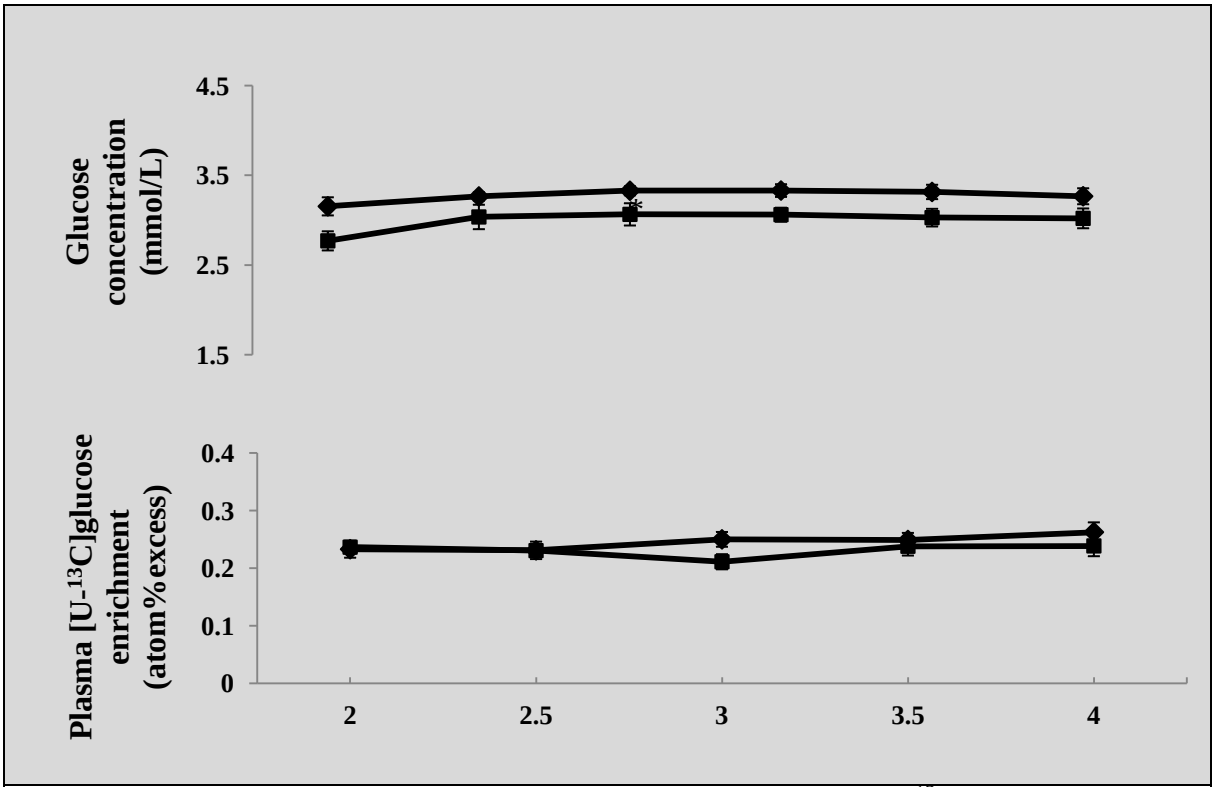


Fig 5.5 Time course changes in plasma glucose concentration and [U-^{13}C]glucose enrichment during the last 2 h of isotope infusion in sheep fed GOCon diet (♦) and Garlic Oil diet(■).

The enrichments of plasma phenylalanine and tyrosine and their concentrations is shown in fig 5.6 and kinetics of plasma phenylalanine and kinetics is presented in Table 5.5. The enrichments of [$^2H_5$]phe, [$^2H_4$]tyr and [$^2H_2$]were stable during the last 2 h of the isotope dilution method. The plasma concentration of phenylalanine and tyrosine were also similar at last 2 h of isotope dilution method. The turnover rates of plasma phenylalanine, tyrosine were also similar between the diets. The phenylalanine hydroxylation to tyrosine also did not differ between the diets. Based on phenylalanine and tyrosine enrichments, the whole body protein synthesis was calculated, which was similar between the diets.

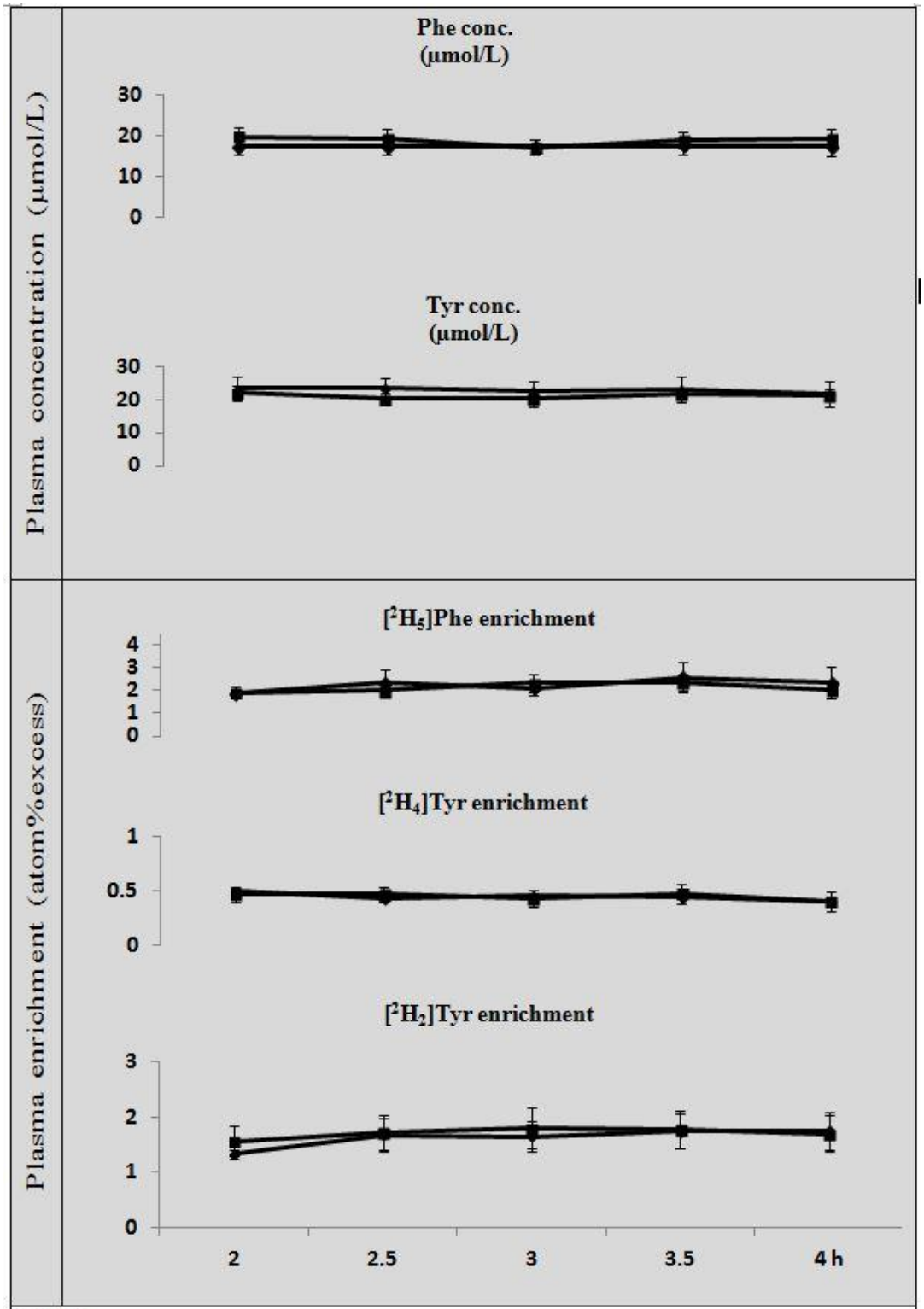


Fig 5.6
Time course change in Plasma Phe and Tyr concentrations and [$^2H_5$]Phe, [$^2H_4$]Tyr, and [$^2H_2$]Tyr enrichments during the last 2 h of isotope infusion in sheep fed GOCon(♦) and Garlic oil diet (■).

Table 5.5 Effects of feeding garlic oil in plasma phenylalanine and tyrosine kinetics in sheep [a].

	Diet [b]			
Items	**GOCon**	**Garlic Oil**	**SEM**[c]	**P-value**
Phe (μmol/L)	17.33	16.90	2.60	0.87
Tyr (μmol/L)	22.79	19.05	3.93	0.36
Phe TR (μmol/kg$^{0.75}$/h)	107.5	121.3	18.83	0.50
Tyr TR (μmol/kgBW$^{0.75}$/h)	139.6	158.7	23.66	0.46
PheOX (μmol/kgBW$^{0.75}$/h)	24.93	23.53	5.16	0.79
WBPS (g/ kgBW$^{0.75}$/d)	9.33	11.06	1.64	0.35

[a] Values represent means of six sheep. [b] GOCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (70:30); Garlic Oil diet: GOCon diet plus Garlic oil@30 mg/kg BW$^{0.75}$/d. [c]SEM, standard error of mean.

The effect of feeding garlic oil in plasma leucine and α-KIC concentration and enrichments of plasma α-[1-^{13}C]KIC is shown in figure 5.7 and plasma leucine turnover rate in Table 5.6. The isotopic enrichments of plasma α-[1-^{13}C]KIC were similar at last 2 h of the isotope dilution method. The plasma leucine and α-concentration did not differ between two diets. The plasma leucine turnover also did not differ between the diets.

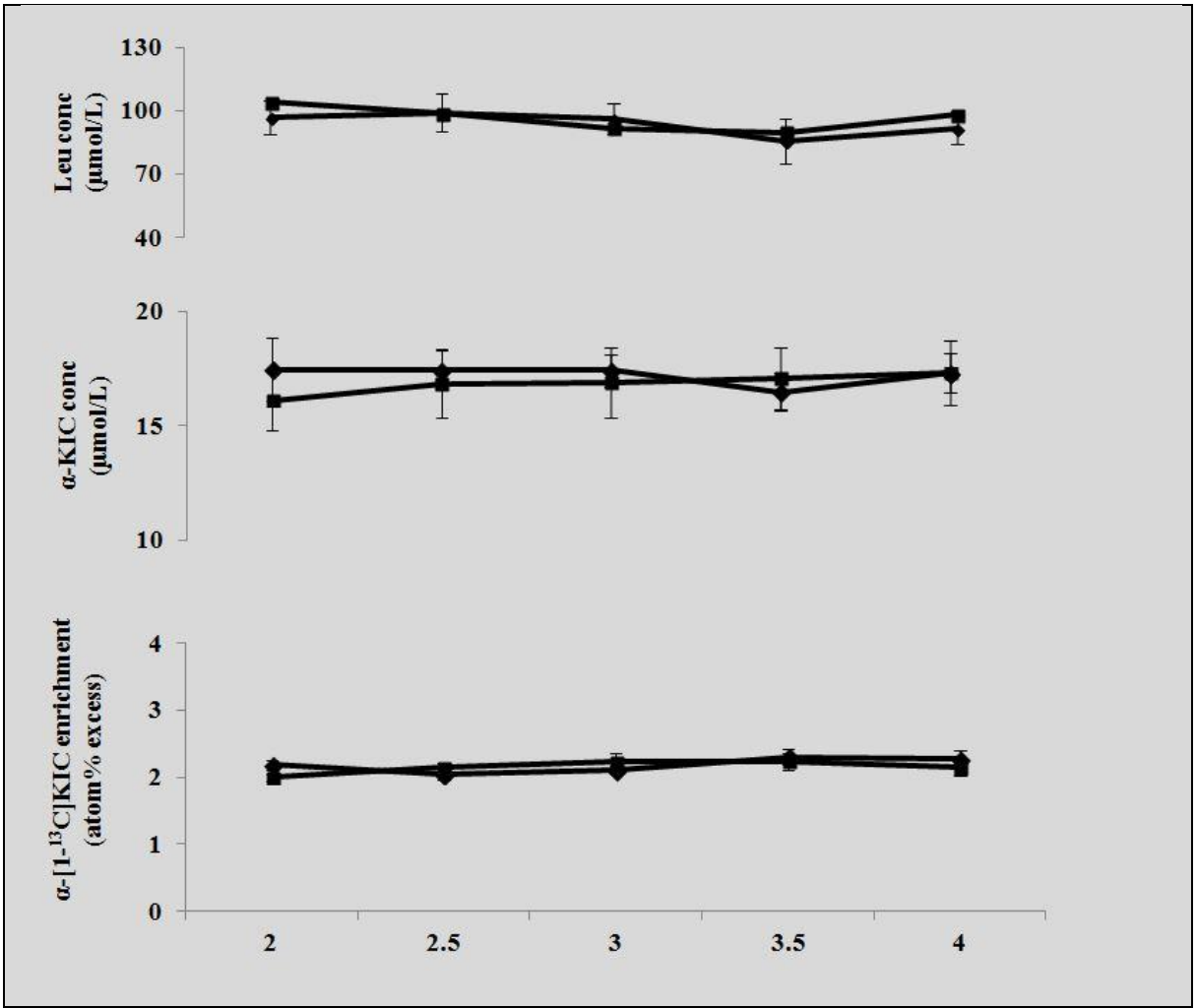


Fig 5.7

Time course change in plasma Leu, α-KIC concentration and α-[1-^{13}C]KIC enrichments during the last 2 h of of isotope infusion in sheep fed GOCon diet (♦) and Garlic Oil diet(■).

Table 5.6 Effects of feeding garlic oil on plasma leucine kinetics in sheep [a].

	Diet [b]			
Items	**GOCon**	**Garlic Oil**	**SEM**[c]	**P-value**
Leu (μmol/L)	93.96	96.58	8.35	0.75
α-KIC (μmol/L)	17.22	16.84	1.42	0.79
Leu TR (mmol/kg$^{0.75}$/h)	0.34	0.39	0.08	0.52

[a] Values represent means of six sheep. [b] GCCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (70:30); Garlic Oil diet: Control diet plus Garlic oil@30 mg/kg BW$^{0.75}$/d. [c]SEM, standard error of mean.

The effect of feeding garlic oil on basal plasma amino concentration is presented in Table 5.7. Plasma amino acid methionine tended to be higher in garlic oil diet. Plasma phenylalanine, serine was also higher in garlic oil diet. Other amino acids did not significantly differ between the diets.

Table 5.7 Effects of feeding garlic oil on plasma AA, NH3 and urea concentration at pre-infusion of isotope in sheep [a].

Item	**Treatment**		**SEM**	**P value**
(μmol/L)	**GOCon diet**	**GO diet**		
Threonine	208.1	219.9	17.53	0.57
Valine	191.4	219.5	13.74	0.17
Methionine	10.9	13.9	0.86	0.07
Isoleucine	63.2	71.1	7.19	0.38
Leucine	106.8	109.9	15.88	0.86
Phenylalanine	47.75	69.4	4.40	0.03
Histidine	50.5	48.8	4.05	0.70
Lysine	91.93	116.2	19.75	0.30
Tryptophan	41.9	38.1	4.48	0.45
Serine	112.8	140.1	8.82	0.04
Aspartic acid	10.9	12.8	2.81	0.54

Glutamic acid	153.1	132.6	41.19	0.65
Glutamine	172.1	217.3	43.97	0.37
Glycine	488.5	542.4	27.00	0.14
Alanine	198.1	180.6	24.87	0.53
Tyrosine	31.5	35.3	2.29	0.23
Arginine	164.6	219.3	26.49	0.13
Proline	92.9	94.1	5.47	0.84
Asparagine	52.1	72.0	10.41	0.19

[a] Values represent means of six sheep. [b] GOCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (70:30); Garlic Oil diet: GOConl diet plus Garlic oil@30 mg/kg $BW^{0.75}$/d. [c]SEM, standard error of mean.

Table 5.8 Effect of feeding garlic oil in purine derivatives excretion and microbial nitrogen supply in sheep [a].

	Diet [b]			
Items	**GOCon**	**Garlic Oil**	**SEM**[c]	**P-value**
Allantoin (mmol/kg $kgBW^{0.75}$/d)	0.36	0.39	0.01	0.177
Uric acid (mmol/kg $kgBW^{0.75}$/d)	0.03	0.04	0.005	0.131
Xanthine plus hypoxanthine (mmol/kg $kgBW^{0.75}$/d)	0.03	0.03	0.003	0.373
Total PD (mmol/kg $kgBW^{0.75}$/d)	0.42	0.46	0.02	0.129
Total MNS (g/kg $kgBW^{0.75}$/d)	0.35	0.38	0.019	0.115

[a] Values represent means of six sheep. [b] GOCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (70:30); Garlic Oil diet: GOCon diet plus Garlic oil@30 mg/kg $BW^{0.75}$/d. [c]SEM, standard error of mean.

The effect of feeding garlic oil on purine derivatives namely allantoin, uric acid, and Xanthine plus hypoxanthine, total purine derivatives and total microbial nitrogen supply in sheep is presented in Table 5.8. There was no effect of garlic oil on allantoin, uric acid, xanthine plus hypoxanthine secretions in urine and thus total microbial nitrogen supply also did not differ between the diets.

The effect of feeding garlic oil on rate of glucose production and utilization and the exogenous glucose infusion during the hyperinslinemic-euglycemic clamp is presented in

Table 5.9. and time course changes were presented in Fig 5.8. No significant differences were observed in the rate of plasma glucose production and utilization as well as amount of endogenous glucose infusion due to garlic oil addition in the diet.

Table 5.9 Effect of feeding garlic oil in rate of glucose production and rate of glucose utilization in sheep [a]

	Diet [b]			
Items	**GOCon**	**Garlic Oil**	**SEM**[c]	**P-value**
Plasma glucose production rate ($mg/kg^{0.75}/min$)	6.37	6.59	0.381	0.534
Plasma glucose utilization rate ($mg/kg^{0.75}/min$)	6.35	6.62	0.382	0.505
Exogenous glucose infusion ($mg/kg^{0.75}/min$)	5.62	5.07	0.417	0.246

[a] Values represent means of six sheep. [b] GOCon diet: mixed hay (orchardgrass and reed canarygrass) and concentrate (70:30); Garlic Oil diet: Control diet plus Garlic oil@30 mg/kg $BW^{0.75}$/d. [c]SEM, standard error of mean.

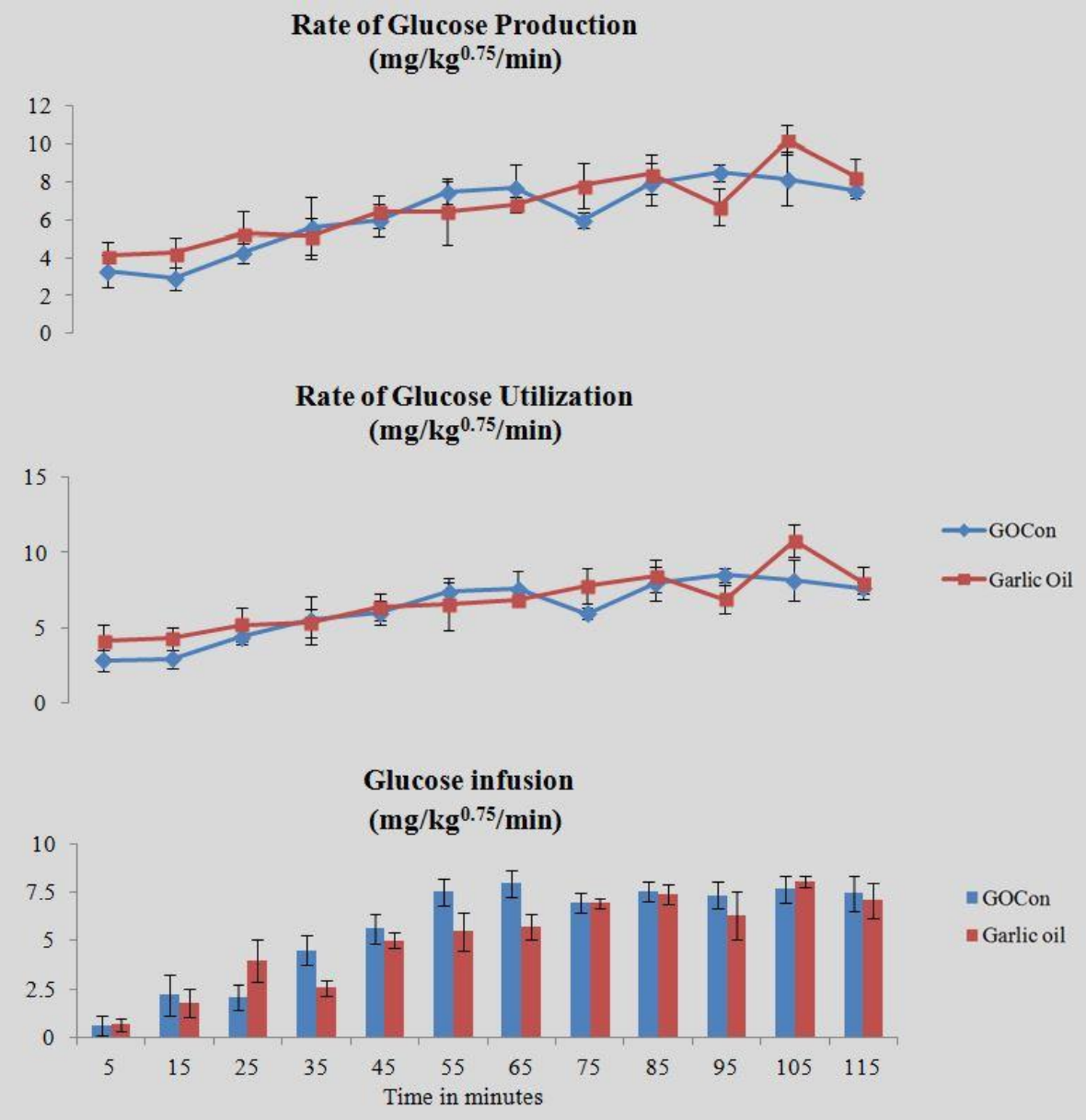


Fig 5.8
Effect of feeding Garlic Oil on plasma glucose production and utilization and exogenous glucose infusion in sheep.

5.4 Discussion

Ruminants establish a symbiotic relationship with rumen microbes and change in pH will affect the ruminal microbes and thus fermentation of feeds, breakdown of fibers and also the synthesis of microbial protein in the rumen (Calsamiglia et al., 2007). Inclusion of garlic oil at 30 mg/kg BW $^{0.75}$/d diet did not affect rumen pH in our study. Rumen pH drops down during excessive fermentation of starch or carbohydrate fraction of the feed or due to disturbance in rumen VFA absorption function. No such impairments were observed in our study on rumen pH. Our earlier experiment using freeze dried garlic leaves and bulb on invitro study and use of garlic leaves on sheep also had no effect on rumen pH. Various studies using garlic oil, garlic leaves and silage, garlic powder or pure garlic constituents by Busquet et al., (2005a); Busquet et al., (2005b); Wanapat et al., (2008); Klevenhusen et al., (2011); Foskolos et al., (2015); Kamruzzaman et al., (2011; Kamruzzaman et al., (2014)on rumen pH had no effect on rumen pH. Zhu et al., (2012) observed decrease in ruminal pH by addition of garlic oil, however the decrease was from 6.55 to 6.49 and stated that such small change would not affect rumen fermentation significantly.

Microorganisms present in rumen degrade nutrients to produce volatile fatty acids as energy supply for ruminants. The effect of addition of garlic oil has been variable in various studies and might be due to the fact that the rumen microbes are adapted or not adapted on those studies. Cardozo et al., (2004) found that garlic oil modified the molar proportions of individual volatile fatty acids between days 2 to 6 of the fermentation in a dual flow continuous culture fermenters system, but the effects were disappeared after 6 day

adaptation. Busquet et al., (2005a; 2005b) also observed change in ruminal VFA concentrations only during the first two days. Difference in adaptation time might be due to different concentrations of garlic oil used in these studies. In our study, animals were adapted to the garlic oil for 14 days prior to the experiment and rumen sample was taken at 21st day of the experimental period thus rumen environment and microbes should have been adapted to the addition of garlic oil. Similar lack of effect was also observed in rumen ammonia concentration. Rumen ammonia is considered to be most important source for microbial protein synthesis in rumen. Our result is consistent with findings of Castillejos et al., (2006), Kamruzzaman et al., (2011), Wanapat et al., (2008), where feeding garlic oil, leaves or powder had no effect on rumen ammonia concentration. Busquet et al., (2005b) found that the effect of garlic oil to be dose dependent as concentrations of 3 to 300 mg/L of garlic oil increased ruminal ammonia concentration whereas higher dose of 3000 mg/L of garlic oil in in-vitro studies decrease ammonia concentration. This might not be applicable in invivo study as ammonia produced in rumen might be under constant vigilance of circulatory system to remove when excess.

The effect of garlic or its compounds in rumen methane emission is contradictory as invitro studies showing no effect (Kamel et al., 2008) to about decrease in methane emission up to 70% by Busquet et al., (2005b) and 91% by Soliva et al., (2011) without significant decrease in apparent organic matter and NDF degradation are available. The reduction in methane emission by Chaves et al., (2008) in lambs, Busquet et al., (2005b) and Soliva et al., (2011) in invitro studies were at 100mg/L and 300 mg/L but assuming rumen volume of 15 L in our sheep the concentration of garlic oil would be about 30mg/L which might be

the reason for not observing the effect of reduction of methane emission. Klevenhusen et al., (2011) however failed to observe decrease in methane emission in sheep at a higher dose of about 500mg/L suggest that the effect of garlic oil in methane emission might be contributed to different experimental conditions and active constituents of garlic oil.

Feeding garlic oil did not increase nitrogen retention in our study even though loss of nitrogen through feces was lower and nitrogen digestibility was higher. Nitrogen retention is considered as an index of protein status in ruminants. Lack of this effect was also observed in microbial nitrogen supply as the purine derivatives were also not different between diets in our study. Hoover and Stokes, (1991) reported that the rate of degradation of carbohydrate is the major factor in controlling microbial growth. As observed in our study lack of change in volatile fatty acid concentration between diets might support the fact that microbial growth and thus microbial protein supply might have been similar in our study.

Propionate is considered to be the major source of glucose in ruminant animal and garlic is also been reported to have potency of increased insulin secretagogue activity (Augusti and Sheela, 1996; Liu et al., 2005). In our study, combined effect of lack of difference in propionate concentration in rumen and effect of garlic oil metabolites might have lowered the basal plasma glucose concentration in our study. The study on use of garlic oil on plasma glucose kinetics using garlic oil is first of its type as no literature is available. We found that addition of garlic oil in diet tended to increase the plasma glucose concentration. The exact mechanism for this is not available however in our earlier experiment using

freeze dried garlic leaves, glucose turnover tended to be increased. This might be because of enhancement of glucose utilization by increasing pancreatic secretion of insulin in animal. Sano et al. (1999) also found that glucose turnover rate increased with increased concentration of insulin in sheep. in the present experiment although insulin concentration was not measured, we speculate that the active sulfur compounds present in garlic might have caused to have higher tendency of glucose turnover. Following the isotopic enrichment experiment, to elucidate the glucose metabolism, we carried out hyperinsulinemic-euglycemic clamp. We however did not observe difference in rate of glucose production and utilization as well as rate of endogenous glucose infusion. This contradictory observation might be due to the fact that the glucose turnover rate was on border line of tendency of significance and increased number of experimental animals might give us a clear understanding.

The effect of plasma amino acids kinetics due to addition of garlic oil were observed using isotopes of leucine, α-KIC, phenylalanine and tyrosine and this is also the first of this type of study as no literature is available. We did not observe difference in plasma amino acid kinetics as nitrogen retention in our study was also not significantly different. Kamruzzaman et al., (2011) carried out study on plasma leucine kinetics in sheep using garlic leaves silage and found no difference in plasma leucine turnover rate although it was numerically higher in garlic leaves diet. Similar study by Kamruzzaman et al., (2014) on plasma phenylalanine and tyrosine using garlic leaves showed that the replacement of garlic leaves at 10% had tendency to increase plasma phenylalanine turnover and whole body protein synthesis. We observed an interesting fact in plasma amino acid concentration that

the basal plasma phenylalanine concentration was higher, but in time course change we did not observe the difference. This might be due to the fact that samples were taken at different time period. Methionine also tended to be higher and serine concentration was also higher in garlic oil diet. Exact mechanism how garlic oil causes to change these amino acid remains still to be investigated.

5.5 Conclusion

From the present findings, it could be concluded that the supplementation of garlic oil had no negative impact on total VFA production as well as methane emission. Garlic oil however improved the nitrogen absorption as well as nitrogen digestibility. Similarly, plasma glucose turnover was also higher in garlic oil diet. No differences were observed in plasma amino acid kinetics as well as rate of glucose production and utilization. Thus, further experiments at different dose rates may help us understand clearly how garlic oil would help in increasing ruminant productivity.

Chapter 6

General discussion and summary

The animal agriculture is a vital part of food industry but it raises environmental concerns due to its contribution on greenhouse gas. Manipulating the rumen ecosystem to enhance the digestibility of fibrous feeds, and reduce methane emission by ruminants to improve animal performance are the major goals for animal nutritionists. Feed additives such as organic acids, ionophores, halogen compound and other antibiotics were used to modify ruminal fermentation, affect ruminal methanogenesis and improve animal performance. However, the use of antibiotics as feed additives due to the concerns of wide spread antibiotic resistant bacteria and residues in dairy and meat products have shifted the research towards use of safe alternatives such as plants containing secondary metabolites. Garlic contains a complex mixture of many secondary metabolites which include allicin, diallyl sulfide, diallyl disulfide and allyl mercaptan. These secondary metabolites present in garlic have been tested for rumen fermentation and methane emission; but studies on use of garlic on plasma glucose, leucine and phenylalanine kinetics have not been studied, thus through a series a experiment, we tried to see the role of garlic on plasma glucose and amino acid kinetics along with rumen fermentation and nitrogen utilization in sheep.

It is well discussed that rate of CH_4 emission are influenced by a range of diet and animal factors, such as feed intake, diet quality, and nutrient utilization efficiency. Thus, an experiment was conducted to see the methane emission in sheep fed iso-energetic diet (100kcal) of mixed hay (MH) (57 $g/kg^{0.75}$/d of feed, 7 $g/kg^{0.75}$/d CP) diet and mixed hay

and concentrate (Conc.) diet (40:60 ratio, 45 g/kg$^{0.75}$/d of feed,) on methane emission, rumen fermentation characteristics, nitrogen utilization, microbial protein synthesis and microbial diversity in rumen of sheep. Experiment was carried out in crossover design of 21 days period with 14 days of adaptation to diet. Conc. diet lowered rumen pH ($P<0.01$). Rumen ammonia, total volatile fatty acid, acetic acid and propionic acid did not differ between the diets however butyric acid was higher ($P<0.01$) in Conc. diet. Acetic to propionic acid ratio was lower ($P=0.01$) in Conc. diet. Total methane emission during the 24 h period did not differ between the diets. The nitrogen intake, fecal nitrogen excretion and urinary nitrogen were higher ($P<0.05$) in MH diet. Nitrogen digestibility was higher ($P<0.01$) in Conc. diet. Urinary derivatives namely allantoin, uric acid, and xanthine and hypoxanthine were higher ($P<0.05$) in Conc. diet and thus total microbial nitrogen supply was also higher ($P<0.01$) in Conc. diet. Microbial diversity measured using Shannon index, Dominance index and Evenness index by DGGE gels did not differ between the diets. Thus, from the present experiment, we can conclude that in iso-energetic diets, concentrate diet have higher nitrogen digestibility and higher microbial nitrogen supply to the animal thus might have positive impact on productivity. Although methane emission and rumen fermentation characteristics did not differ did not differ between the diets, concentrate diet might be beneficial from environmental aspect due to less loss of nitrogen into the environment as nitrogen retention from both the diets was comparable in an iso-energetic condition. Better utilization of nitrogen in diet might can be correlated to faster body growth and thus lesser emission of methane.

In our second experiment, we tried to find if secondary metabolites present in garlic could influence the rumen fermentation, methane emission and dry matter digestibility. The experiment was carried out using Rumen Simulation Technique (RUSITEC) as invitro methods are easy, cheap and faster to compare multiple samples. Control (Con.) diet was 15 g DM of mixed hay (orchardgrass and reed canarygrass). Four levels of freeze dried garlic leaves (10, 20, 40% and 60% DM respectively added to Con. diet) and four levels of freeze dried garlic bulbs (3, 6, 12, 18% DM respectively added to Con. diet). Rumen pH did not differ between the diets. Total VFA was lower ($P<0.05$) in 40% and 60% garlic leaves added diets and 12 % and 18% garlic bulb added diets. Ammonia concentration did not differ between the diets except for 40% garlic leaves added diet tended to be higher ($P=0.07$). Dry matter digestibility lower ($P<0.05$) in 20%, 40%, 60% garlic leaves added diets and 6%, 12%, 18% garlic bulb added diets. Total methane collected was lower ($P<0.05$) in 20%, 40% and 60% garlic leaves added diets and 12% and 18% garlic bulb added diets. Methane collected per g DMD was lower ($P<0.05$) in 20%, 40% and 60% garlic leaves added diet and 12 % and 18 % garlic bulb added diets. In the present experiment, garlic leaves or bulb at higher concentration lowered methane emission which was desirable but consequently lowered the dry matter digestibility also which was undesirable, thus garlic leaves (around 10 to 20%) or bulb (3% to 6%) concentration seems to be appropriate and more experiments using garlic at these lower concentration could give us appropriate dose to use as a diet additive based on this study on the parameters studied.

Although in-vitro methods are cheap, easier and faster, but its significance cannot be validated until they are further verified on in-vivo conditions, thus in the third experiment

we fed garlic leaves to sheep to see the effects on rumen fermentation, methane emission, plasma glucose kinetics and nitrogen utilization in sheep. Six sheep were fed freeze dried garlic leaves at 2.5 g/kg $BW^{0.75}$/d (about 5% DM of diet) (FDGL diet) added to control diet using a crossover design. Control diet (GLCon. diet) consisted of mixed hay and concentrate at 60:40 ratio. Plasma glucose turnover rate was measured using primed continuous infusion of [U-^{13}C]glucose. No significant differences in rumen fermentation parameters were noticed except for rumen ammonia tended to be higher for FDGL diet. Methane emission per animal per day did not differ between the diets but methane emission per kg dry matter ingested and methane emission per kg dry matter digested was lower ($P<0.05$) for FDGL diet. Plasma glucose concentration was similar between the diets but plasma glucose turnover rate tended ($P<0.10$) to be higher in FDGL diet. Nitrogen intake was higher ($P<0.01$) in FDGL diet due to extra N available due to addition of garlic leaves. Fecal nitrogen was lower ($P<0.05$) in FDGL diet and thus nitrogen absorption was higher ($P<0.01$). Nitrogen retention and nitrogen digestibility were also higher ($P<0.05$) in FDGL diet. Purine derivatives namely allantoin tended ($P<0.10$) to be higher in FDGL diet and thus total microbial nitrogen supply also tended ($P<0.10$) to be higher in FDGL diet. Inclusion of FDGL as feed supplement had no negative effects on ruminal fermentation characteristics and had positive N utilization. However, further research seems necessary to explain its potential on methane reduction as well as glucose turnover.

In the fourth experiment, we examined the effect of feeding garlic oil to sheep on rumen fermentation characteristics, methane emission, nitrogen utilization, plasma glucose, leucine, phenylalanine and tyrosine kinetics. Primed continuous infusion of [U-^{13}C]glucose,

[1-^{13}C]leucine, [$^{2}H_5$]phenylalanine and [$^{2}H_2$]tyrosine were carried out followed by hyperinsulinemic euglycemic clamp. Control (GOCon.) diet consisted of mixed hay and concentrate (70:30) and experimental (GO) diet consisted of garlic oil added at 30 mg/kgBW$^{0.75}$/d to GOCon diet. Rumen pH, ammonia, VFA concentration and methane emission did not differ between the diets. Glucose concentration was lower ($P<0.05$) in GO diet and glucose turnover rate was higher ($P<0.05$) in GO diet. Nitrogen intake did not differ between the diets. Fecal nitrogen was lower ($P<0.05$) in GO diet and thus nitrogen absorption was higher ($P<0.05$) in GO diet. Urinary nitrogen excretion did not differ between the diets and thus nitrogen retention also was similar between the diets. The leucine, phenylalanine and tyrosine turnover rates did not differ within the diets. The whole body protein synthesis and degradation based on phenylalanine tyrosine model did not differ between the diets. The hyperinsulinemic euglycemic clamp did not differ between two diets in rate of glucose production and utilization and exoogenous glucose infusion despite the lower blood glucose concentration and tendency of increased glucose turnover rate. Based on these the effect of garlic oil on ruminant feedstuff use is still debatable and further studies are necessary.

Based on the above experimental findings we can thus conclude that garlic leaves, garlic bulb or garlic oil failed to mitigate methane emission; and positive effects on nitrogen utilization and glucose metabolism are still debatable. Feeding garlic leaves or garlic oil in ruminants could be a potential feed additive in future, however further research is recommended.

ニンニク添加飼料給与ヒツジにおけるメタン放出および血漿栄養素代謝

畜産は農業の重要な分野であるが、温室効果ガス(GHG)への寄与による環境に対する懸念が増大している。反芻家畜は消化吸収器官である第一胃を持ち、第一胃内に棲息する微生物による発酵作用により本来ほ乳動物が利用できないセルロース等の栄養素を利用できる。しかし、この過程でメタンを生成して大気中に放出してしまうため、メタン放出の抑制は解決すべき重要な課題である。これまで、イオノフォアや抗生物質などの飼料添加物は第一胃発酵を変化させて動物の成績を向上させるために使用されてきた。しかしながら、広範な耐性菌や畜産物への残留の懸念から飼料添加物としての抗生物質は二次代謝産物を含む植物のような安全な代替物の研究に移行してきた。ニンニクは allicin、diallyl sulfide、diallyl disulfide および allyl mercaptan など多くの二次代謝産物を含んでいる。ニンニクの二次代謝産物は第一胃発酵やメタン放出の研究で使用されているが、血漿グルコース、ロイシン、フェニルアラニン、チロシンに関する代謝に及ぼすニンニクの影響は研究されていない。したがって、一連の研究を通じて我々はヒツジにおいて第一胃発酵性状、窒素出納とともに血漿グルコースおよびアミノ酸代謝に及ぼすニンニクの役割を明らかにしようとした。

【実験１】

メタン放出量は飼料摂取量、飼料の品質、栄養素の利用効率のような要因の影響を受ける。そこで、ヒツジを用いてメタン放出量に及ぼす給与飼料の影響を明らかにしようとした。飼料は混播乾草(MH 飼料、57 g/kg$^{0.75}$/日)および混播乾草と濃厚飼料の混合飼料(粗濃比 40:60、Conc 飼料、45 g/kg$^{0.75}$/日)とし、いずれも ME エネルギー給与量を 100 kcal/kg$^{0.75}$/日とした。Conc. 飼料は第一胃 pH を低下させた($P < 0.01$)。第一胃アンモニア、全 VFA、酢酸およびプロピオン酸濃度は飼料間に差がなかったが、酪酸濃度、酢酸：プロピオン酸比は Conc.飼料が高かった($P < 0.01$)。24 時間のメタン放出量は飼料間に差がなかった。窒素摂取量、糞中および尿中窒素排泄量は MH 飼料で高かった($P < 0.05$)。窒素消化率および微生物態窒素供給量は HC 飼料で高かった($P < 0.01$)。DGGE 測定による第一胃内微生物叢は飼料間に差がなかった。以上の結果から、濃厚飼料は窒素消化率および微生物態窒素供給量が高いため、生産性に好影響をもたらすと結論される。メタン放出量および第一胃発酵性状は飼料間に差が認められなかったが、濃厚飼料は環境への窒素損失が少ないことから有益であるかもしれない。飼料の優れた窒素利用は高成長やメタン放出量の削減と関連しているのかもしれない。

【実験２】

ニンニクに存在する二次代謝産物が第一胃発酵性状、メタン放出量や DM 消化率に影響を与えるか否かを明らかにしようとした。実験には多数のサンプルを迅速、簡便かつ精度

高く比較できる *in vitro* の実験装置である Rumen Simulation Technique (RUSITEC)を使用した。対照飼料(Con. diet)は混播乾草とした。ニンニクの添加量は凍結乾燥したニンニク茎葉(10、20、40、60%飼料乾物(DM))とニンニク球根(3、6、12、18%飼料 DM)とした。第一胃 pH は飼料間で差がなかった。総 VFA は 40、60%茎葉添加飼料、12、18%球根添加飼料が Con. 飼料より低かった($P < 0.05$)。アンモニア濃度は飼料間に差がなかった。DM 消化率は 20、40、60%茎葉添加飼料および 6、12、18%球根添加飼料で低かった($P < 0.05$)。メタン生成量および可消化 DM あたりのメタン生成量は 20、40、60%茎葉添加飼料および 12、18%球根添加飼料で低かった($P < 0.05$)。以上の結果から、高水準の茎葉あるいは球根では DM 消化率の低下に伴いメタン生成量が低下することが示された。

【実験３】

in vitro で得られた結果は *in vivo* と必ずしも一致しない。そこで、実験３では凍結乾燥したニンニク茎葉を添加した飼料をヒツジに給与し、第一胃発酵性状、メタン放出量、血漿グルコース代謝動態および窒素利用性を明らかにしようとした。ヒツジ 6 頭を用い、対照 (GLCon. diet) 飼料は混播牧草と濃厚飼料の給与比率を 60:40 とした。GLCon. 飼料に 2.5g/kg$^{0.75}$/日の凍結乾燥ニンニク茎葉(FDGL 飼料、5%飼料 DM)を給与した。血漿グルコース代謝回転速度は[U-^{13}C]グルコースの同位元素希釈法を用いて測定した。第一胃発酵性状は飼料間に差が認められなかった。１頭あたりのメタン放出量は飼料間に差はなかったが、DM 摂取量あたりおよび DM 消化量あたりのメタン放出量は FDGL 飼料で低かった($P < 0.05$)。血漿グルコース代謝回転速度は FDGL 飼料で高い傾向を示した($P < 0.10$)。窒素蓄積は GL 飼料で高く($P < 0.05$)、微生物態窒素供給量は高い傾向を示した($P < 0.10$)。したがって、飼料へのニンニク茎葉添加はメタン放出量を低下させ、窒素蓄積を増加させることにより環境に好影響を与え、炭水化物代謝を促進することが示唆された。

【実験４】

ガーリックオイルは活性含硫物質の割合が高く、容易に入手できるのためガーリックオイルを用い、ヒツジの飼料利用性、第一胃発酵性状、メタン放出量、栄養素(グルコース、ロイシン、フェニルアラニン、チロシン)代謝動態およびインスリン作用を明らかにしようとした。ヒツジ 6 頭を用い、対照(GOCon)飼料は混播牧草と濃厚飼料の給与比率を 70:30 とした。GOCon 飼料に 30mg/kg$^{0.75}$/日のガーリックオイル(GO 飼料)を投与した。血漿グルコース、ロイシン、フェニルアラニン、チロシン)代謝動態は同位元素希釈法([U-^{13}C]グルコース、[1-^{13}C]ロイシン、[$^{2}H_5$]フェニルアラニン、[$^{2}H_2$]チロシン)、インスリン作用はグルコースクランプ法により測定した。第一胃 pH、アンモニア、VFA 濃度およびメタン放出量は飼料間に差がなかった。血漿グルコース濃度は GO 飼料が低かったが($P < 0.05$)、血漿グルコース代謝回転速度は高かった($P < 0.05$)。糞中窒素排泄量は GO 飼料が低く($P < 0.05$)、窒素吸収量は高かった($P < 0.05$)。血漿アミノ酸代謝回転速度および血漿フェニルアラニン

とチロシンの代謝動態から算出した全身のタンパク質合成速度は飼料間に差がなかった。GO 飼料においてグルコースクランプ法実施中における血糖値は低く、血漿グルコース代謝回転速度は低い傾向を示したにもかかわらず、グルコース利用速度およびグルコース注入速度は差がなかった。したがって、反芻家畜におけるガーリックオイルの使用には議論の余地があり、さらなる研究が必要である。

【結論】

本実験で得られた結果から、ニンニク茎葉、ニンニク球根あるいはガーリックオイルはメタン放出を抑制しないが、家畜の生産性を改善する窒素利用およびグルコース代謝には有効であると結論された。したがって、ニンニクは将来的に反芻家畜における有力な飼料添加物になり得ることが示された。

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