Responses of Feeding Garlic Stem and Leaf on Plasma Nutrients Metabolism in Ruminants

The United Graduate School of Agricultural Sciences

Graduate School of Iwate University

Science of Bioproduction

(Iwate University)

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Responses of Feeding Garlic Stem and Leaf on Plasma Nutrients Metabolism in Ruminants

A dissertation

Submitted to the committee of United Graduate School of Agricultural Sciences, Iwate University, Japan in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

By

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September, 2012

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*I am myself **Md. Kamruzzaman** is declaring that the dissertation with above mentioned title presented herein for the degree of Doctor of Philosophy is the result of my own experiments. The references from other works used for my information are highly acknowledged.*

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Md. Kamruzzaman

The dissertation with above mentioned title presented herein for the degree of Doctor of Philosophy is approved as to style and content by

.....

Professor, Hiroaki SANO, PhD
(Major Supervisor)

Contents

Contents

Chapter-3

Chapter-4

Dedication:

This dissertation is dedicated to my Mother for her untold patience, selfless sacrifice and infinite encouragements to open every single gate of success.

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Md. Kamruzzaman

September, 2012

Abbreviations:

AFRC:	Agricultural and Food Research Council
AMP:	Adenosine 5-phosphate
BW:	Body weight
CP:	Crude protein
DM:	Dry matter
GC/MS:	Gas chromatography mass spectrometry
IMP:	Inosine 5-phosphate
α-KIC:	α-ketoisocaproic acid
Leu:	Leucine
ME:	Metabolizable energy
MNS:	Microbial nitrogen supply
mPhe	α-methyl-phenylalanine
MPS	Microbial protein synthesis
MTBSTFA:	*N-tert.*-butyldi-methylsilyl-*N*-methyltrifluoroacetamide
mTyr:	α-methyl-tyrosine
NDF:	Neutral detergent fiber
NEFA:	Non-esterified fatty acids
NPN:	Non-protein nitrogenous substances
PD:	Purine derivatives
PheOX:	Phenylalanine oxidation
Phe:	Phenylalanine
SSA:	Sulfosalicylic acid

Tyr:	Tyrosine
VBN:	Volatile basic nitrogen
WBPD:	Whole body protein degradation
WBPF:	Whole body protein flux
WBPS:	Whole body protein synthesis
XO:	Xanthine oxidase

General Introduction:

Antibiotic growth promoters have been widely used in animal industry for decades worldwide to prevent diseases and metabolic disorders, as well as to improve the efficiency of converting feed materials to varied animal products for humans. But in recent years, the use of antibiotics in animal feeds is facing reduced social acceptance due to the increasing public concerns about the bad residual effects of antibiotics on human health. Since January, 2006, European parliament restricted the use of antibiotics as animal feeds in the European Union (OJEU, 2003). Consequently, animal scientists are seeking alternative additives that would improve feed efficiency and animal productivity by altering its metabolism favorably. In the last decade, various natural plants and their extracts have received increased attention as most promising alternatives to antibiotics for animal industry (Cardozo et al., 2004; Busquet et al., 2006; Bodas et al., 2008).

Garlic (*Allium sativum*) as flavoring spice and folk medicine has been used since antiquity in almost every known culture (Rivlin, 2001). Garlic contains many sulphur-containing compounds which are partly responsible for health beneficial effects of garlic (Amagase et al., 2001). The major sulphur-containing compounds in intact garlic are γ-glutamyl-*S*-allyl-L-cysteins and *S*-allyl-L-cysteine sulphoxides (alliin) which are converted into thiosulphinates (such as allicin) via enzymic reactions when raw garlic is processed (Amagase, 2006). When garlic stem and leaf is cut or ruptured, alliin is rapidly converted to allicin. After ingestion, allicin further is converted to other sulfur compounds like diallyl sulfide, diallyl disulfide and diallyl trisulfide in the rumen or in the liver. Garlic bioactive components are well known to possess antiviral (Weber et al., 1992; Ankri and Mirelman, 1999), antifungal (Yoshida et al., 1987; Feldberg et al.,

1988), antiparasitic (Ankri and Mirelman, 1999; Amagase et al., 2001), antioxidant (Prasad et al., 1995; Ide et al., 1997), anticancer (Amagase and Milner, 1993; Agarwal, 1996), hypocholesterolemic (Jain et al., 1993; Yeh and Liu, 2001), liver functioning (Amagase, 2006) and immune enhancing (Amagase et al., 2001; Amagase, 2006) properties in many human and animal studies. Effects also have been reported that garlic bioactive components improve the efficiency of nutrient use by decreasing energy (in the form of methane) or N (in the form of ammonia) in continuous rumen culture (Cardozo et al., 2005; Busquet et al., 2006; Kamel et al., 2008). Garlic constituents have been used in the diet of ruminants (Cardozo et al., 2005; Busquet et al., 2006; Kamel et al., 2008), chickens (Qureshi et al., 1983a, 1983b; Yalcin et al., 2006), pigs (Horton et al., 1991; Qureshi et al., 1987; Chen et al., 2008) and some laboratory animals (Chi et al., 1982; Yeh and Liu, 2001; Bruck et al., 2005) in limited scale.

In ruminants, most of the fermentation of feed particles occurs in rumen. The rumen is described as large fermentation vat which contains a variety of different microorganisms which act synergistically to break down feed particles for the host animal. After extensive fermentation by the resident microbes, fermentation products mainly volatile fatty acids (VFA) from carbohydrates and microbial protein from protein and non-protein nitrogenous substances become available for the host. In ruminants, little glucose is absorbed from the digestive tract and must be obtained via gluconeogenesis.

During harvesting period garlic bulb yields a considerable amount of stem and leaf which is simply thrown or disposed. Garlic stem and leaf are greenish materials, edible, have a characteristic spicy flavor and are rich in protein value. It also contains allicin, the key bioactive component of garlic (Mohsen and Shahab, 2010). It is

expected that these residues could be used as ruminant feed due to its abundant bioactive components which ultimately will facilitate the proper use of the renewable residues as well as reduce feed scarcity for ruminants. However, to date, no information is available on potential growth promoting effects of garlic stem and leaf as ruminant feed. It was hypothesized that bioactive components of garlic stem and leaf might affect nutrients metabolism, without resulting in overall inhibition of rumen fermentation in sheep. Therefore, I was interested to conduct a couple of researches to evaluate the feeding effects of garlic stem and leaf in sheep. Initially, ruminal fermentation characteristics, N balance, turnover rates (TR) of plasma leucine (Leu), whole body protein synthesis (WBPS) and whole body protein degradation (WBPD) were determined using garlic stem and leaf silage. Thereafter, microbial nitrogen supply (MNS), TR of plasma phenylalanine (Phe), tyrosine (Tyr), acetate and glucose and WBPS and WBPD were determined using fresh garlic stem and leaf in the succeeding studies.

Chapter-1

Effects of feeding garlic stem and leaf silage on ruminal fermentation characteristics, nitrogen balance, rates of plasma leucine turnover and whole body protein synthesis in sheep

Introduction:

It has been reported that garlic components influenced the fermentation characteristics in the *in vitro* studies (Cardozo et al., 2005; Busquet et al., 2006; Kamel et al., 2008). Garlic components also resulted in lower proportion of ruminal acetate and higher proportions of propionate and butyrate (Busquet et al., 2005a, 2005b). It was also found that garlic constitutes increased protein anabolism and decreased protein catabolism in rats (Oi et al., 2001). However, until recently, no information is available on potential growth promoting effects of garlic stem and leaf on ruminal fermentation characteristics and N metabolism in ruminants. It was hypothesized that bioactive components of garlic stem and leaf might affect whole body N metabolism without interfering ruminal fermentation in ruminants by enhancing digestive and metabolic functions in the body. Garlic stem and leaf materials could be conserved easily by the process of ensiling for a long time. Therefore, considering the above features, the current study was undertaken to evaluate the feeding effects of garlic stem and leaf silage on ruminal fermentation characteristics and N metabolism in sheep using [1-^{13}C]leucine isotope dilution technique and N balance test.

Materials and methods:

The study was conducted from July to October, 2009 at Faculty of Agriculture,

Iwate University, Japan. Animal care procedures and protocol was approved and followed according to the guidelines established by the Animal Care Committee of Iwate University.

Sheep, diets and experimental protocol

Sound, healthy four crossbred (Corriedale x Suffolk) shorn wethers, averaging 47.5±2.2 kg of body weight (BW) at the beginning of the study, were used. Two different dietary treatments, one was mixed hay (Hay-diet, CP 11.9%, NDF 64.8%; on air dry matter basis) of orchardgrass (*Dactylis glomerata*) and reed canarygrass (*Phalaris arundinacea*) and another one (GS-diet) was that where 10% of hay was replaced by garlic stem and leaf silage (CP 9.3%, NDF 48.7%; on air dry matter basis) were tested. The amount of hay was calculated according to Agricultural and Food Research Council (AFRC, 1993) at maintenance ME level. The animals were received 8.8 g CP and 120 kcal ME/kg $BW^{0.75}$/d for the Hay-diet. The experiment was performed using a crossover design with two 21 days periods. Two sheep were fed GS-diet during the first period and then Hay-diet during the second period but other two were fed in the reverse sequence. Sheep were fed twice a day at 08:30 and 20:30 h and had free access to water. On day 15, sheep were moved to controlled house and maintained in wooden metabolism stalls designed for total collection of faeces and urine. The sheep were housed in individual pens in a sheep barn during the adjustment period (first two weeks) and then moved to environmental controlled house at an air temperature of 23℃, 70% relative humidity and with lighting from 08.00 to 22.00 h. The sheep were weighed on the day of starting experiment and every 7 days intervals.

Preparation of garlic stem and leaf silage

During harvesting period, garlic stem and leaf were collected from selected farmers

at Aomori Prefecture in Japan. Prior to processing, the dead and dirty stem and leaf were removed. The cleaned plants were chopped at a particular size (around 6-8 cm) and mixed properly. The chopped materials were then kept into plastic silo pits (180 L), pressed sufficiently to make anaerobic condition and filled properly. After one month of processing, silo was opened and silage was offered to animals. Before feeding the animals, chemical composition and some basic fermentation parameters of garlic stem and leaf silage were detected accordingly (Table 1.1).

Table 1.1 Fermentation profile of garlic stem and leaf silage.

Parameters	Value
Physical properties:	
Color	clean, greenish
Moulds/fungus	ND[a]
Taste	pleasing
Chemical properties:	
Moisture (%)	82
pH	4.2
Lactic acid (g/kg DM[b])	47
Acetic acid (g/kg DM)	16
Isobutyric acid (g/kg DM)	ND
Butyric acid (g/kg DM)	ND
Lactic: acetic acid	2.9
VBN[c] (g/kg total N)	64

[a] ND, not detected; [b] DM , dry matter; [c] VBN , volatile basic nitrogen.

Collection of faeces, urine and rumen fluid

Nitrogen balance was conducted over five successive days of the last week of each 21 days treatment.

A

B

B

B

Figure 1.1. A: Garlic stem and leaf cultivated at Aomori Prefecture, Japan; B: Offering garlic stem and leaf silage to sheep.

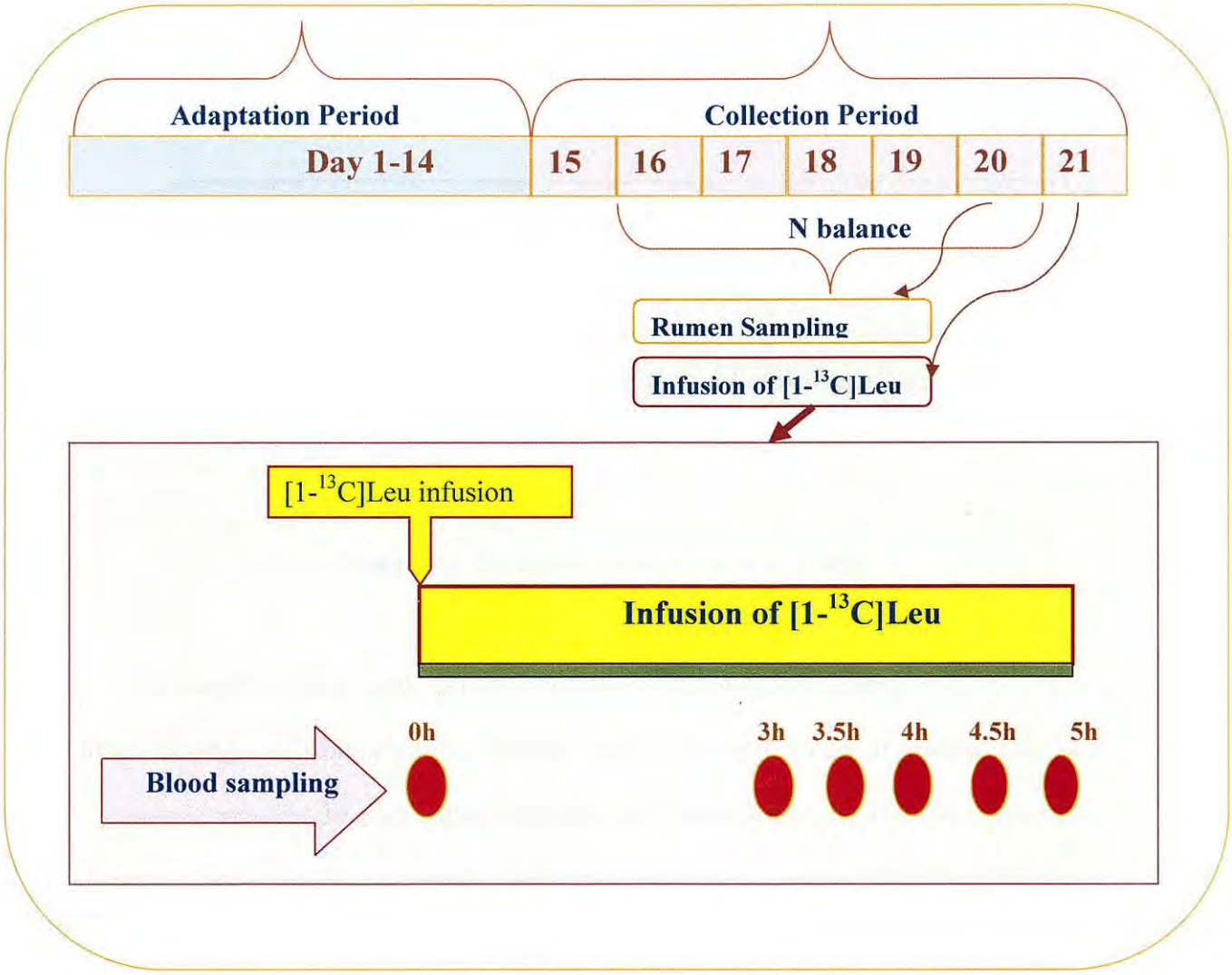

Figure 1.2. Experimental layout and [1-^{13}C]Leu infusion method.

Urine was collected in plastic buckets containing 50 mL of 6N H_2SO_4 solution to prevent N loss, the total volume was recorded, thawed properly and subsamples (50 mL) was stored at -30℃ until further analysis. Faeces were collected for each 24 h, oven dried (60℃, 48 h) and placed at room temperature for five days. Then the air dried samples was weighed and ground to pass through a 1 mm screen and sub-samples were kept into plastic containers and stored until further analysis.

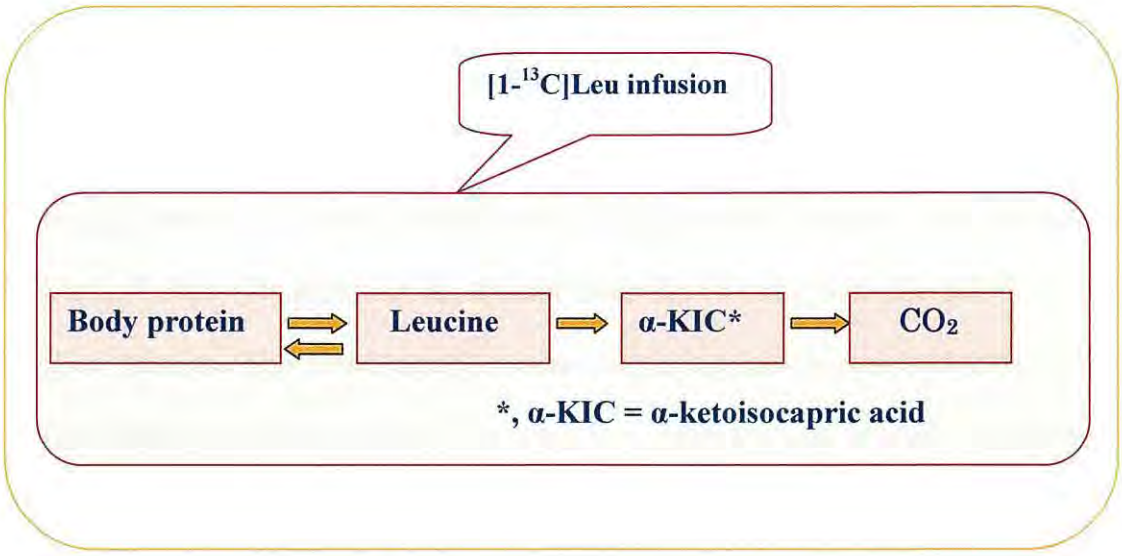

Figure 1.3. Leucine turnover model in sheep.

On day 20 of each trial, rumen fluid was collected before feeding and at 3 and 6 h after feeding via stomach tube inserted orally. The pH value of rumen fluid was measured immediately after collection with a pH meter (MH-10P, Toa Electronics Ltd., Japan). A portion of it was centrifuged at 8000 rpm for 10 min at 2℃ (RS-18IV, Tomy, Japan) and supernatant (2 mL) was acidified with HCl for NH_3 analysis. The remaining portion was used for the analysis of rumen VFA concentrations. Finally, both the samples were kept at -30℃ for further analysis.

Isotope dilution method

An isotope dilution method using [1-^{13}C]Leu was carried out on day 21 of each dietary treatment to determine Leu and protein metabolism in sheep. Experimental layout and isotope dilution method is shown in Figure 1.2 and Figure 1.3. Two catheters, one for isotope infusion and another for blood sampling, were inserted into the left and right jugular veins on the morning of isotope dilution method. At 12.00 h, on the respective day, [1-^{13}C]Leu (4 mmol/L in 0.9% of sodium chloride solution,

L-leucine-1-^{13}C, minimum 99 atom% excess ^{13}C; Cambridge Isotope Laboratories, Inc., USA) was continuously infused by a multichannel peristaltic pump (AC-2120, Atto, Japan) at a rate of 7.2 μmol/kg $BW^{0.75}$/h for 5 h via jugular infusion catheter, preceded by a priming dose of 7.2 μmol/kg $BW^{0.75}$ of [1-^{13}C]Leu. Blood samples were collected immediately before (10 mL) and at 30 min intervals (5 mL) over the last 2 h of [1-^{13}C]Leu infusion. The blood samples were transferred into heparinized tubes and stored temporarily in ice box until centrifugation. After the end of the experiment, plasma was harvested from blood cells by centrifugation at 8000 rpm for 10 min at 2℃ and an aliquot was stored at -30℃ for further analysis.

Laboratory analyses

Nitrogen contents in diets, faeces and urine were analyzed by Kjeldahl method with the Foss Kjeltec System (Tecator Digestor System and Kjeltec, 2300, Foss Tecator, Sweden; Figure 1.4). Neutral detergent fibre (NDF) in diets was determined according to Van Soest et al. (1991) using Foss Analytical FiberCap™ 2021/2023 system. The pH of garlic silage fluid was measured by the pH meter. The lactic acid concentration of garlic silage was determined according to the procedure described by Taylor (1996). Volatile fatty acids concentration of garlic silage fluid was determined using a gas chromatography (HP-5890, Hewlett Packard, USA; Figure 1.5). Volatile basic N content of garlic silage was also determined (Dhaouadi et al., 2007). The NH_3 content of the rumen fluid was determined by colorimetric method (Weatherburn, 1967). Rumen urea was measured using a urea kit (Urea NB, Wako Pure Chemical Industries Ltd., Japan). Volatile fatty acids concentrations in rumen fluid were determined using the gas chromatography. In brief, raw rumen fluid was centrifuged (0℃, 8000 rpm, 10 min) and 5 mL supernatant was taken in VFA glass tube, added with 1 g magnesium sulphate

($MgSO_4.7H_2O$) and 1 mL of 20% H_2SO_4. Then the tube was set with distillation apparatus, resulted steam distillate was collected into a glass beaker and titrated with 0.1N NaOH. The titrated distillate volume was reduced first with open flame and then dried with oven (60℃) and finally molar percentage of individual VFA were determined using the gas chromatography acidifying with 1 mL of 5N H_3PO_4 solution.

A B

Figure 1.4. Kjeldahl method with the Foss Kjeltec System (Tecator Digestor System and Kjeltec, 2300, Foss Tecator, Sweden; A: digestion set, B: distillation & titration set).

Plasma free amino acids, NH_3 and urea concentrations were determined using an automatic amino acid analyser (JLC-500/V, JEOL, Japan; Figure 1.6). In brief, 1 mL of blood plasma was added with 1 mL of 3% sulfosalicylic acid (SSA) and kept into refrigerator for overnight. Then, it was centrifuged (4℃, 3000 rpm, 15 min) twice and

supernatant was transferred to another tube. Finally, supernatant was filtered through 0.45 μm HV filter (Toyo Roshi Kaisha Ltd., Japan) and transferred to vials for determining plasma free amino acids, NH_3 and urea concentrations.

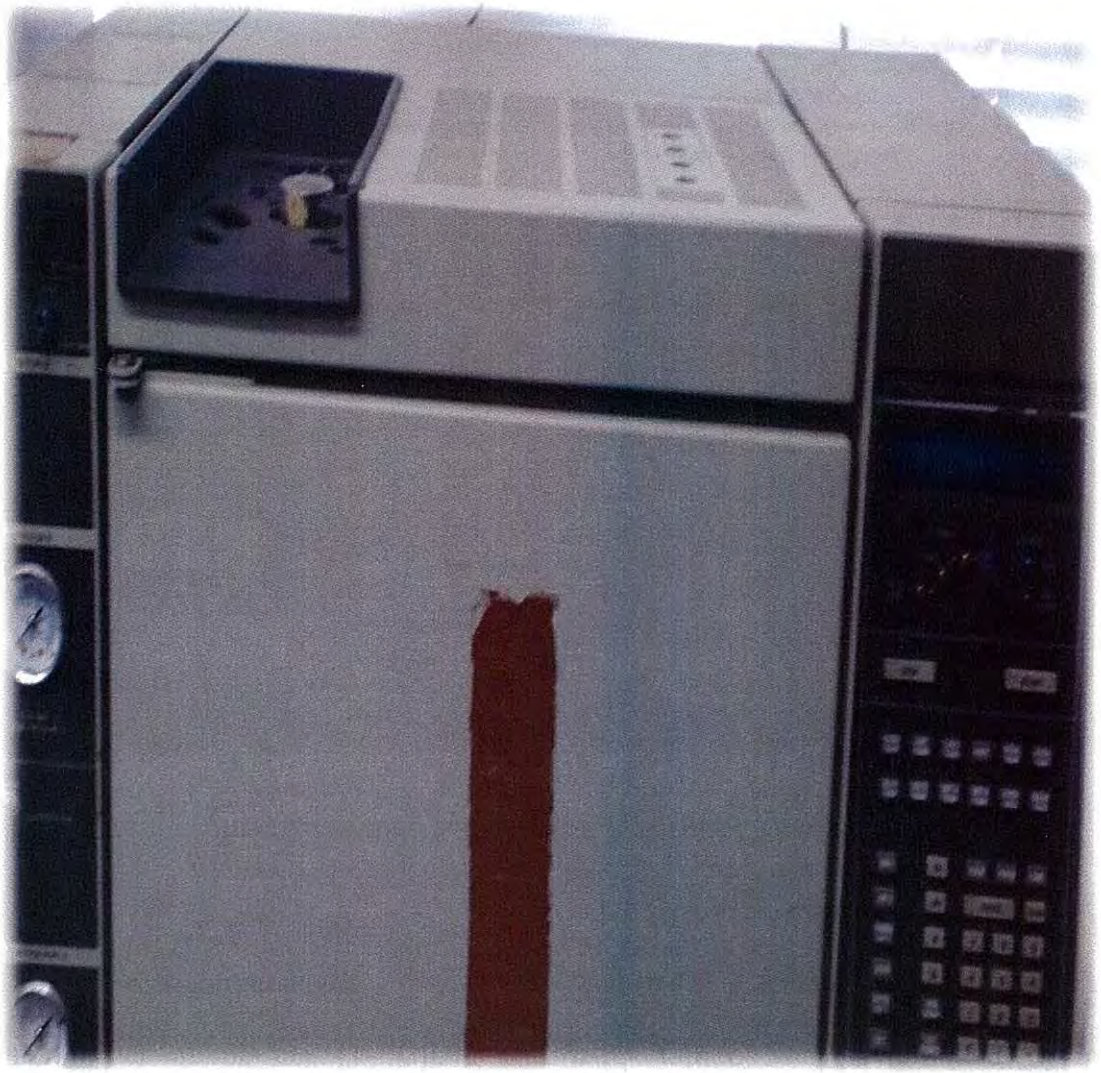

Figure 1.5. Gas chromatography (HP-5890, Hewlett Packard, USA; the injector and detector temperature 170℃; oven temperature elevated from 100℃ to 165℃ by carrier gas (He) flow at 1.0 mL/min).

Concentration of plasma glucose was determined enzymatically by the method of

Huggett and Nixon (1957). Concentrations of plasma non-esterified fatty acids (NEFA) were also enzymatically determined using a kit (NEFA C, Wako Pure Chemicals Industries Ltd., Japan).

Figure 1.6. Automatic amino acid analyser (JLC-500/V, JEOL, Japan).

The concentrations of plasma Leu and α-ketoisocaproic acid (α-KIC) and enrichments of plasma [1-^{13}C]Leu and α-[1-^{13}C]KIC were determined by gas chromatography mass spectrometry (QP-2010, Shimadzu, Japan; Figure 1.7) with selection ion monitoring according to the procedures of Rocchiccioli et al. (1981) and Calder and Smith (1988) as described previously by Sano et al. (2004). Briefly, 1 mL blood plasma sample was mixed with 1 mL of 4% SSA, 100 μL of n-leucine (0.5 mmol/L) and 100 μL of ketoisovaleric acid (0.05 mmol/L) properly and kept into

refrigerator for 30 min. Then, the samples were centrifuged (0℃, 12000 rpm, 10 min) twice and upper transparent supernatant was transferred to another tube. Thereafter, the supernatant was poured to column consisted of 0.5 mL of cation exchange resin (Dowex 50 X8 (H^+ form, 200-400 mesh), washed with 0.5 mL distilled water twice and eluant was kept for α-KIC analysis. Then, again the column tube was set on another new plastic tube, washed with 1 mL distilled water, added with 1 mL of 4N NH_4OH twice and washed finally with 1 mL distilled water. Then, 0.5 mL of eluant was transferred into screw capped glass tube and kept into dessicator for Leu analysis. For α-KIC, 1 mL of obtained eluant was taken into screw capped glass tube, mixed with 0.5 mL of 1% o-phenyendiamine hydrochloric acid solution (4 mol/L) and heated for 1 h at 90℃. After cooling for 1 h at normal temperature, 2 mL ethyl-acetate was added and mixed vigorously for 1 min and centrifuged (4℃, 1000 rpm, 10 min). Then, the supernatant was dried with anhydrous Na_2SO_4 for 2 h and finally supernatant was taken into screw capped glass tube and dried with N_2 gas. After drying, both Leu and α-KIC samples were mixed with 25 μL of acetinitrile and *N-tert.*-butyldi-methylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA), heated at 80℃ for 20 min, transferred into chromatographic vials and enrichments were measured by gas chromatography mass spectrometry with selection ion monitoring. The ions determined are: m/z 302 and 303 for Leu, m/z 245 for KVA and m/z 259 and 260 for KIC, respectively.

Calculations of plasma LeuTR, WBPS and WBPD

Plasma Leu turnover rate (LeuTR) was calculated using the following equation described by Wolfe (1984).

$$\text{LeuTR [mmol/kg BW}^{0.75}\text{/h]} = I \times (1 / E - 1)$$

where, I is the infusion rate of [1-^{13}C]Leu isotope and E is the plasma isotopic

enrichments of α-[1-^{13}C]KIC during the steady state. In the present study, plasma enrichment of α-[1-^{13}C]KIC was used to determine the LeuTR as because α-[1-^{13}C]KIC is the true precursor of intracellular Leu metabolism reported by Sano et al. (2004) in their previous study.

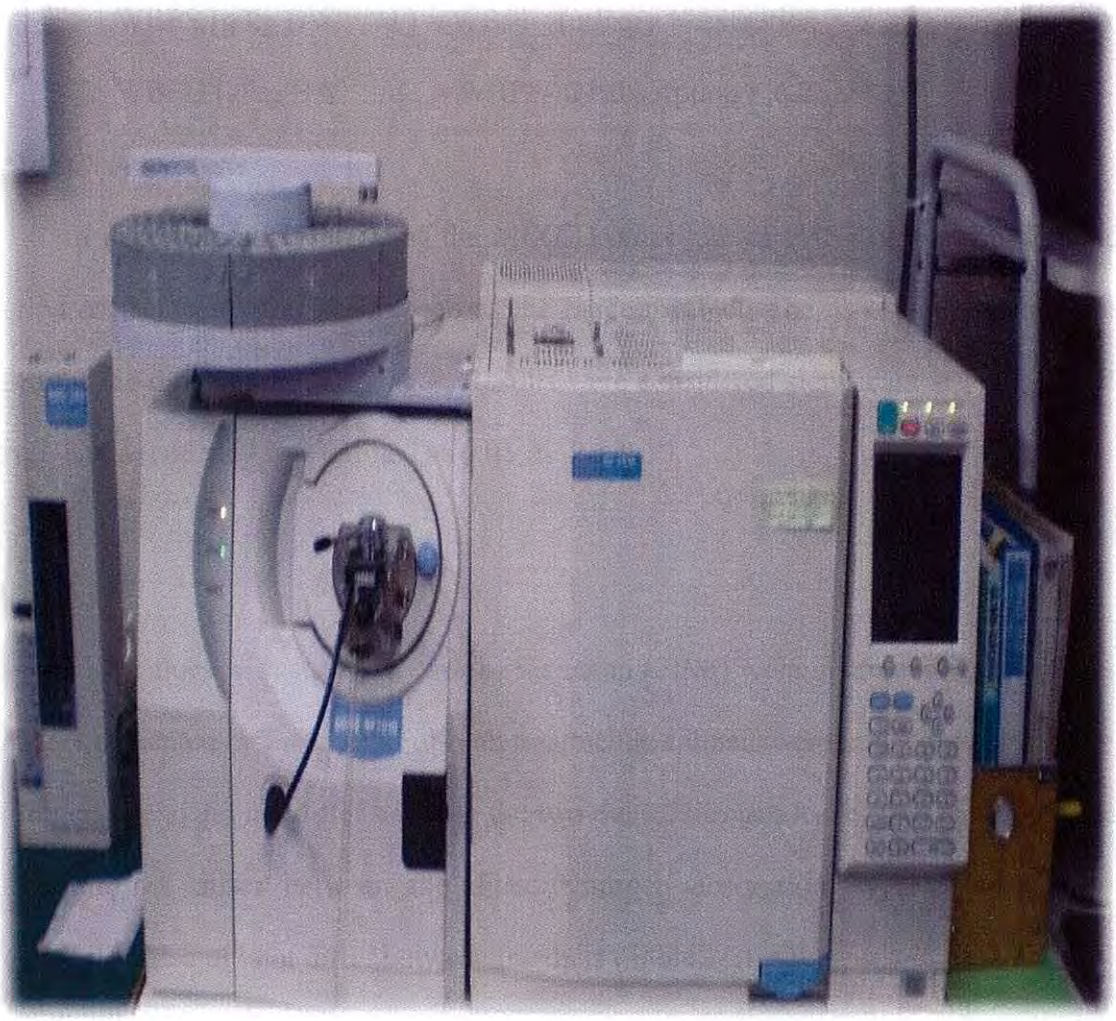

Figure: 1.7. Gas chromatography mass spectrometry (QP-2010, Shimadzu, Japan). Column HP-1, Column temperature 150℃ for 6 min, 35℃/min, 300℃ for 5 min; Inlet temperature 275℃; Interface and ion mobile phase temperature 250℃.

This result was converted to whole body protein flux (WBPF) by dividing 0.066;

the value 0.066 represents the carcass concentration of Leu in sheep as described by Harris et al. (1992). Then, WBPS and WBPD were determined from the relationships described by Schroeder et al. (2006) as follows:

$$\text{WBPF [g/kg BW}^{0.75}\text{/d]} = \text{LeuTR} / 0.066$$

$$\text{WBPS [g/kg BW}^{0.75}\text{/d]} = \text{WBPF- (urinary N excretion} \times 6.25)$$

$$\text{WBPD [g/kg BW}^{0.75}\text{/d]} = \text{WBPF- (N absorption} \times 6.25)$$

Statistical analysis

All data were analysed using the MIXED procedure of SAS (1996) with diet and period as the fixed effect and sheep as the random effect. The Tukey adjustment was used for the time course of changes. Results were considered significant at the $P < 0.05$ level, and a tendency was defined as $0.05 \leqq P < 0.10$.

Results:

Mean values with standard error of the mean (SEM) were given. Nitrogen balance data are presented in Table 1.2. For both treatments, animals were in positive N balance. Nitrogen intake did not differ between the two diets. Nitrogen losses through faeces and urine did not differ between the diets. Nitrogen absorption, N retention and N digestibility also did not differ between the diets (Table 1.2).

Time course of changes in rumen pH and principal VFA concentrations are shown in Figure 1.8. Average ruminal fermentation patterns are presented in Table 1.3. Rumen pH was almost same and rumen NH_3 and urea did not differ between the diets. Rumen total VFA concentration as well as concentrations of principal VFA did not differ between the diets. Acetate to propionate ratio also did not differ between the diets.

Of the plasma free amino acids determined at the pre-infusion period of the isotope

dilution method, concentrations of plasma threonine, valine, isoleucine, leucine, histidine and alanine were significantly lower ($P = 0.01$, $P = 0.01$, $P = 0.04$, $P < 0.01$, $P = 0.04$ and $P < 0.01$ respectively) in GS-diet than Hay-diet (Table 1.4). Plasma glucose concentration was significantly greater ($P = 0.03$) and NEFA concentration was lower ($P < 0.01$) for GS-diet compared with Hay-diet (Table 1.4).

Table 1.2 Effects of feeding garlic stem and leaf silage on N absorption, N retention and N digestibility in sheep[a].

Items	Treatment[b]		SEM[c]	*P*- Value
	Hay- diet	GS - diet		
N intake (g/kg $BW^{0.75}$/d)	1.40	1.39	0.01	0.59
N in faeces (g/kg $BW^{0.75}$/d)	0.51	0.47	0.02	0.22
N in urine (g/kg $BW^{0.75}$/d)	0.51	0.52	0.01	0.75
N absorption (g/kg $BW^{0.75}$/d)	0.88	0.92	0.01	0.35
N retention (g/kg $BW^{0.75}$/d)	0.37	0.39	0.01	0.62
N digestibility (%)	64	66	1	0.26

[a] Values represent means of four sheep.

[b] Hay-diet, mixed hay of orchardgrass and reed canarygrass hay;
GS-diet, hay plus garlic silage (at a ratio of 9:1).

[c] SEM, standard error of the mean.

Time course of changes in plasma Leu, α-KIC concentration and α-[1-^{13}C]KIC enrichment during 180 to 300 min of primed continuous infusion of α-[1-^{13}C]Leu

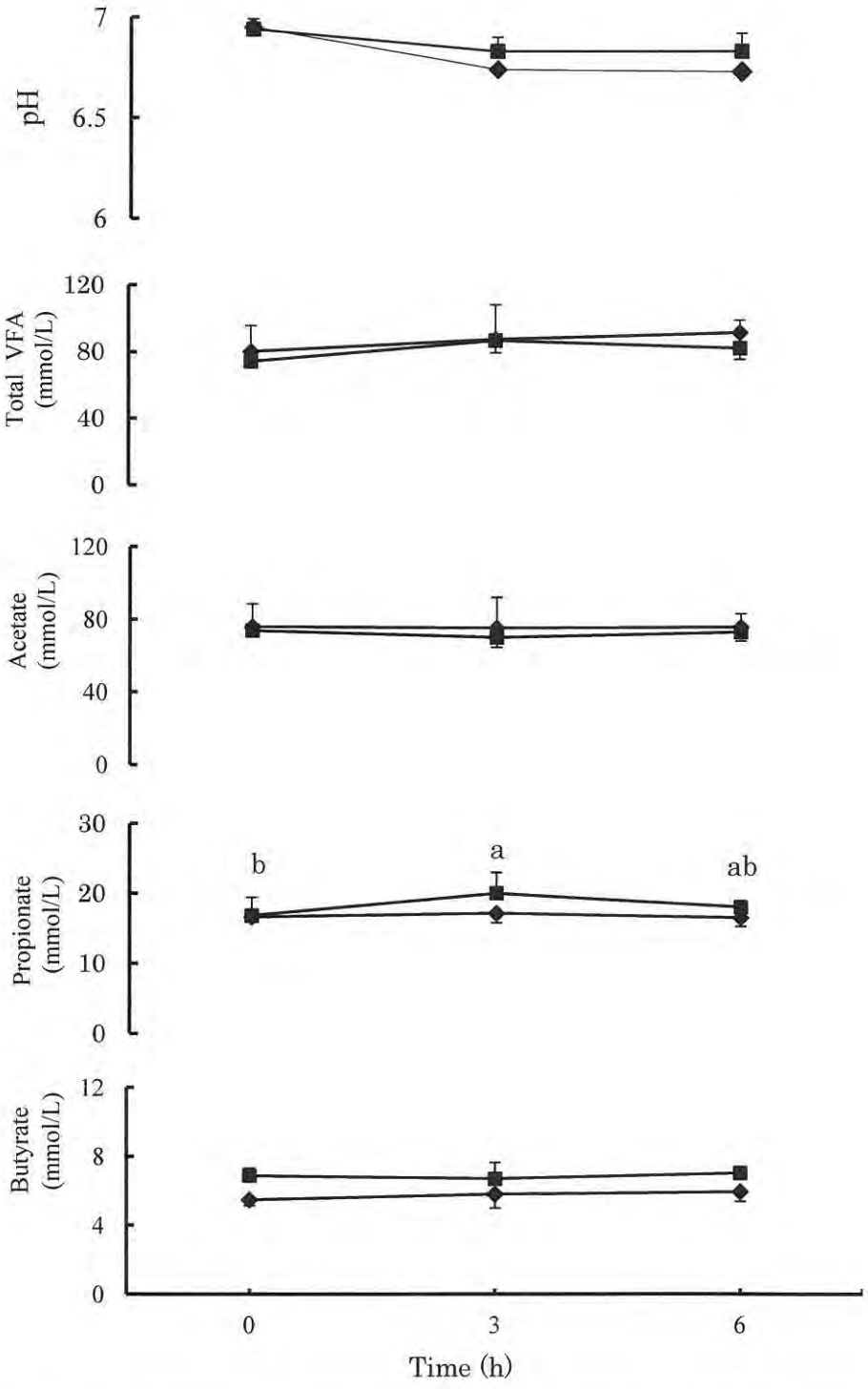

Figure 1.8. Time course of changes in rumen pH and VFA concentrations in sheep fed two different diets (Hay-diet, ◆ and GS-diet, ■).

a, b- differ significantly ($P < 0.05$).

Table 1.3 Effects of feeding garlic stem and leaf silage on ruminal fermentation patterns in sheep[a].

Items	Treatment[b]		SEM[c]	*P*-value
	Hay-diet	GS-diet		
pH	6.81	6.87	0.04	0.22
NH_3 (mmol/L)	10.3	9.1	0.6	0.14
Urea (mmol/L)	3.7	3.5	0.2	0.11
Total VFA (mmol/L)	86.5	81.1	6.4	0.53
Individual VFA (mmol/L)				
Acetate	65.4	58.5	5.5	0.33
Propionate	14.5	14.9	0.9	0.77
Isobutyrate	0.5	0.6	0.03	0.26
Butyrate	4.9	5.9	0.3	0.29
Isovalerate	0.6	0.7	0.1	0.06
Valerate	0.4	0.5	0.03	0.11
Acetate: propionate	4.4	3.9	0.2	0.13

[a] Values represent means of four sheep.

[b] Hay-diet, mixed hay of orchardgrass and reed canarygrass hay; GS-diet, hay plus garlic silage (at a ratio of 9:1).

[c] SEM, standard error of the mean.

in sheep is also demonstrated in Figure 1.9., indicating that plasma α-KIC concentration and α-[1-^{13}C]KIC enrichment reached almost plateaus during the latter periods of the isotope dilution method for each treatment. Plasma Leu metabolism, WBPS and WBPD data are given in Table 1.5. Plasma Leu concentration determined during the later periods of the isotope dilution method, was tended to be lower (P = 0.05) and plasma α-KIC concentration was significantly lower (P = 0.01) in GS-diet than Hay-diet. Plasma LeuTR, WBPS and WBPD did not differ between the diets.

Table 1.4 Effects of feeding garlic stem and leaf silage on plasma free amino acids, ammonia, urea, glucose and NEFA concentrations at the pre-infusion period in sheep[a].

Items	Treatments[b]		SEM[c]	*P*-value
	Hay-diet	GS-diet		
Essential amino acids (μmol/L)				
Threonine	264	212	25	0.01
Valine	302	238	18	0.01
Methionine	22	21	2	0.30
Isoleucine	102	84	7	0.04
Leucine	146	112	7	<0.01
Phenylalanine	55	58	5	0.44
Histidine	57	45	4	0.04
Lysine	106	91	12	0.20
Non essential amino acids (μmol/L)				
Serine	168	170	13	0.95
Asparagine	50	43	5	0.23
Glutamic acid	128	86	11	0.10
Glutamine	260	263	21	0.92
Glycine	525	484	44	0.47
Alanine	217	172	16	<0.01
Tyrosine	83	65	8	0.13
Tryptophan	51	56	5	0.65
Arginine	148	141	17	0.65
Proline	115	95	11	0.19
Ammonia (μmol/L)	296	114	49	0.10
Urea (mmol/L)	7.62	6.36	0.89	0.16
Glucose (mmol/L)	3.18	3.36	0.11	0.03
NEFA (μEq/L)	107	74	11	<0.01

[a] Values represent means of four sheep.

[b] Hay-diet, mixed hay of orchardgrass and reed canarygrass hay;
GS-diet, hay plus garlic silage (at a ratio of 9:1).

[c] SEM, standard error of the mean.

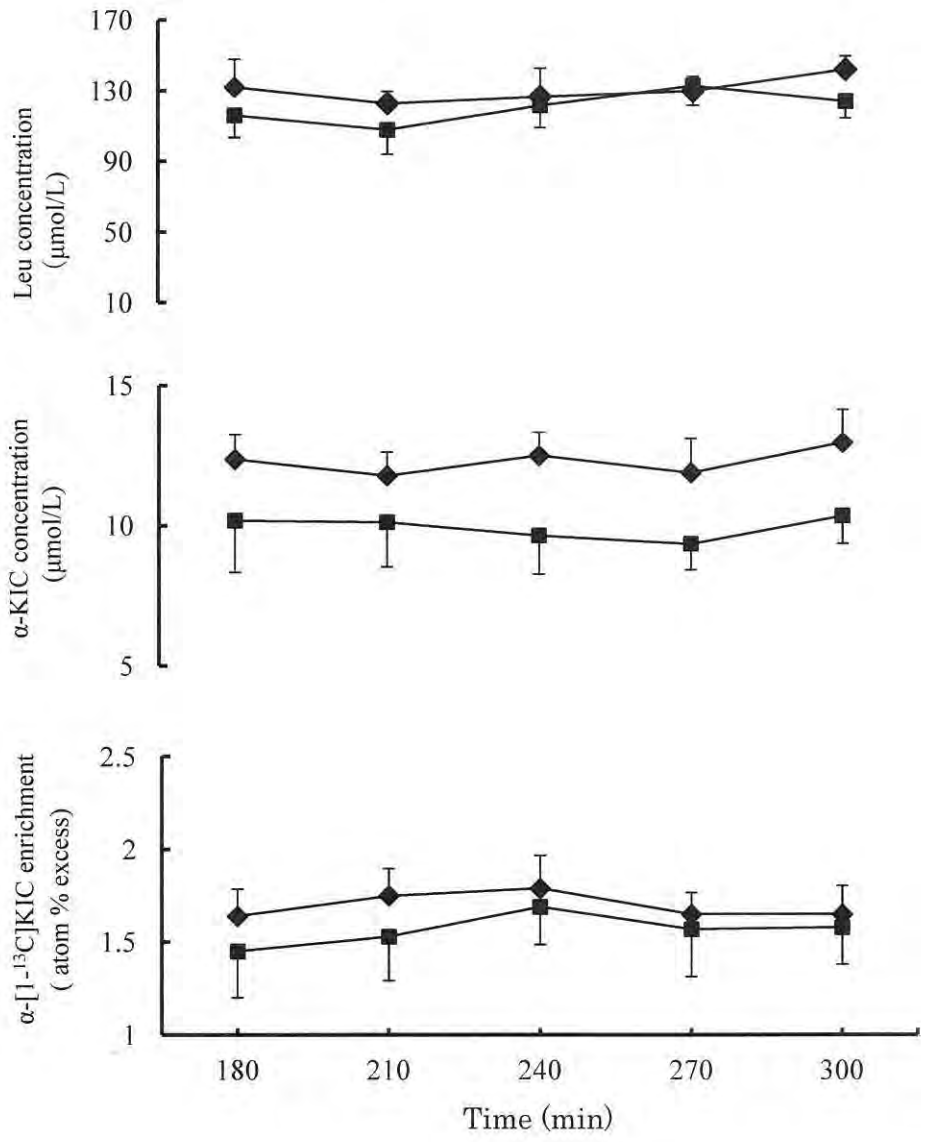

Figure 1.9. Time course of changes in plasma Leu, α-KIC concentration and α-[1-^{13}C]KIC enrichment during 180 to 300 min of primed continuous infusion of α-[1-^{13}C]Leu in sheep fed two different diets (Hay-diet, —◆— and GS-diet, —■—).

Discussion:

Nitrogen retention is considered as an important index of protein status in ruminants. In the present study, N digestibility, N absorption and N retention were comparable

between the two diets. The values of N balance in GS-diet correspond with those of Horton et al. (1991) who investigated the effect of garlic powder and reported that garlic supplementation did not affect apparent digestibility of organic matter and crude protein in lambs. Besides, garlic powder supplementation improved rumen ecology and nutrients digestibility in sheep in another report (Wanapat et al., 2008). The inconsistency of our present findings with Wanapat et al. (2008) might be partly related to garlic components varying in bioactive components because the author used garlic powder and in the present study garlic stem and leaf silage were used.

Table 1.5 Effects of garlic stem and leaf silage feeding on LeuTR, WBPS and WBPD in sheep[a].

	Treatment[b]			
Items	Hay-diet	GS-diet	SEM[c]	*P*-value
Leu (μmol/L)	128	117	6	0.05
α-KIC (μmol/L)	12.3	9.9	0.8	0.01
LeuTR (mmol/kg $BW^{0.75}$/h)	0.37	0.42	0.02	0.11
WBPS (g/kg $BW^{0.75}$/d)	14.5	16.6	0.8	0.14
WBPD (g/kg $BW^{0.75}$/d)	12.2	14.1	0.8	0.13

[a] Values represent means of four sheep.

[b] Hay-diet, mixed hay of orchardgrass and reed canarygrass hay;
GS-diet, hay plus garlic silage (at a ratio of 9:1).

[c] SEM, standard error of the mean.

Inclusion of garlic stem and leaf silage in this trial did not affect the total as well as individual VFA concentrations in sheep which agreeing with Cardozo et al. (2004), who

examined various natural plant extracts on ruminal fermentation profiles and reported that garlic extracts (0.22 mg/L, 0.7% allicin) supplementation had no effects on ruminal total VFA concentration or the proportions of acetate, propionate or butyrate in continuous rumen culture. Nevertheless, Busquet et al. (2005a, 2005b) examined the effect of garlic oil in a continuous culture system and reported that garlic supplementation significantly decreased the proportion of acetate and increased the proportions of propionate and butyrate compared with the control.

Plasma glucose concentration was significantly greater ($P = 0.03$) for GS-diet than Hay-diet which showed that garlic constituents might influence the responses to glucose metabolism in sheep fed with garlic stem and leaf silage. The elevated plasma glucose concentration for GS-diet also might be due to the fact that gluconeogenesis process was enhanced for GS-diet. Furthermore, plasma glucose concentration was not changed by garlic supplementation in the diet of rat in a previous report (Chi et al., 1982). Plasma NEFA concentration was significantly lower ($P < 0.01$) for GS-diet compared to Hay-diet. Plasma NEFA are considered a biomarker of negative energy balance, where the supply of energy is insufficient to meet the needs. In a previous study, it was reported that plasma NEFA is the best indicator of body lipid loss (Sano et al., 1999). Lower plasma NEFA concentration in GS-diet might be due to the reduction of lipolysis as well as increase of fatty acid oxidation in sheep fed garlic stem and leaf silage. Hence, the lower plasma NEFA value for GS-diet confirmed that components of garlic silage might play role in reducing stress and ensure energy balance on animals fed garlic stem and leaf silage. It is revealed that concentrations of plasma threonine, valine, isoleucine, leucine, histidine and alanine at the pre-infusion period were lower in GS-diet than Hay-diet. Lower plasma amino acids concentrations for GS-diet might be due to the

effect that incorporation of such amino acids in protein synthesis in sheep was higher in GS-diet than Hay-diet. Lower amino acids concentration for GS-diet might be the result of gluconeogenesis in GS-diet.

Until now, to our knowledge, the feeding effects of garlic constituents on amino acids kinetics, WBPS and WBPD have not been investigated clearly either in humans or in animals and data are not available. Al-Mamun et al. (2008) supplemented plantain herb (*Plantago lanceolata*) having antimicrobial and antioxidative properties, in the diet of sheep and reported that WBPS was not influenced largely by plantain supplementation. Oi et al. (2001) concluded that garlic supplementation might enhance protein anabolism and suppress protein catabolism by increasing plasma luteinizing hormone (LH) and testicular testosterone and decreasing plasma corticosterone in rats fed a high protein diet. In our present study, WBPS were comparable between the diets which is not consistent with Oi et al. (2001). It might be related with type and dose of garlic components varying in bioactive components because the author used garlic powder which might contain higher amount of bioactive components than garlic stem and leaf.

Chapter-2

Effects of feeding garlic stem and leaf on microbial nitrogen supply, turnover rates of plasma phenylalanine and tyrosine and whole body protein synthesis in sheep

Introduction:

In our previous work (Chapter -1), garlic stem and leaf silage were used and found that garlic silage did not affect ruminal fermentation characteristics in sheep without giving any clear indications of N metabolism. In the previous findings, N retention and LeuTR were numerically higher in GS-diet and it was seemed that garlic stem and leaf might affect microbial protein synthesis in sheep which is considered as in important protein status index in ruminants. For the initial study, garlic stem and leaf were conserved by the process of ensiling. It was assumed that ensiling might cause losses of some nutrients and bioactive components of garlic stem and leaf. It was also assumed that fresh garlic stem and leaf might be more palatable for the animals than the silage. For this reason, fresh garlic stem and leaf were used instead of garlic stem and leaf silage in the present study. Furthermore, to better understand the effects of garlic stem and leaf on N metabolism, the current study was undertaken to evaluate the feeding effects of garlic stem and leaf on MNS, turnover rates of plasma Phe and Tyr (PheTR, TyrTR) and WBPS in sheep using different technique to determine N metabolism in sheep. In this study, [$^2H_5$]Phe model was used for the above purpose.

Materials and methods:

The study was conducted from July to September, 2010, using the individual

feeding barn, environmental controlled house at Faculty of Agriculture, Iwate University, Japan. Experimental protocol was submitted, approved and followed according to the guidelines established by the Animal Care Committee of Iwate University.

Animals, diets and management

Four crossbred (Corriedale x Suffolk) adult shorn crossbred wethers, weighing 55.2±3.3 kg BW were used. Two different dietary treatments were tested. One group received mixed hay (Hay-diet: CP 10.4%, NDF 61.2%, on air DM basis) of orchardgrass (*Dactylis glomerata*) and reed canarygrass (*Phalaris arundinacea*) and another group received the hay diet where 10% of hay DM was replaced by garlic stem and leaf (GL-diet: CP 10.6%, NDF 59.5%, on air DM basis). The amount of hay was calculated according to Agricultural and Food Research Council (AFRC, 1993) at maintenance ME level. The animals were received 7.7 g CP and 120 kcal ME/kg $BW^{0.75}$/d for the Hay-diet. The diets were prepared daily and feed was offered twice a day at 09:30 and 18:30 h and had free access to water. During harvesting period (on July, 2010), garlic stem and leaf were collected from selected farmers at Aomori Prefecture in Japan and stored at -30℃ into room freezer. Before feeding, garlic stem and leaf were kept into normal room temperature for defrosting, cut into small pieces and offered to the animals. The sheep were housed in individual pens in a sheep barn during the adjustment period (first two weeks) and then moved to environmental controlled house at an air temperature of 23℃, 70% relative humidity and with lighting from 08.00 to 22.00 h and maintained in wooden metabolism stalls designed for total collection of faeces and urine for 5 days. The experiment was performed using a crossover design with two 21 days periods in which either Hay-diet or GL-diet were tested. Two sheep was fed GL-diet during the first period and then Hay-diet during the second period but

other two were fed in the reverse order. An isotope dilution method using [$^2H_5$]Phe and [$^2H_2$]Tyr was conducted on day 21 of each dietary treatment to determine N kinetics in sheep.

Collection of faeces and urine

Daily collection of faeces and urine was made between 16 and 20 days of each period. Faeces were collected from each sheep for each 24 h, dried in a forced air oven at 60℃ for 48 h, ground to 1 mm mesh and stored until further analysis. Urine was also collected from each sheep for 24 h in plastic buckets containing 100 mL of 10% H_2SO_4 solution, the total volume was recorded, thawed properly and subsamples (50 mL) was taken in plastic bottle for further processing. From the subsample, 4 mL of urine was taken into another plastic bottle and diluted with water by 5 times to prevent occurrence of precipitation (particularly of uric acid) for determining purine derivatives (PD) excretion. The remaining portion of subsamples was used to determine N content in urine. Finally both the samples were kept at -30℃ until later chemical analyses. The mechanisms of PD excretion are shown in Figure 2.1 and Figure 2.2.

Isotope dilution method

On day 21 of each dietary treatment, an isotope dilution method of [$^2H_5$]Phe and [$^2H_2$]Tyr was conducted to determine the kinetics of plasma Phe, Tyr and WBPS in sheep. Experimental layout and isotope dilution method are shown in Figure 2.3 and Figure 2.4. Cannulation and blood sampling were done avoiding unnecessary stress to the animals. Two catheters, one for isotope infusion and another for blood sampling, were inserted into the both jugular veins on the morning of the 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 3.0 μmol/kg $BW^{0.75}$ of

[$^2H_5$]Phe (L-phenylalanine, ring-D_5, 98% ; Cambridge Isotope Laboratories, Inc., USA), 1.8 μmol/kg $BW^{0.75}$ of [$^2H_4$]Tyr (L-4-hydroxyphenyl-2,3, 5, 6-D_4-alanine, 98 atom % ; Isotec Inc., A Matheson, USA Co., USA) and 1.8 μmol/kg $BW^{0.75}$ of [$^2H_2$]Tyr (L-tyrosine, ring-3,5-D_2, 98% ; Cambridge Isotope Laboratories, Inc., USA) was injected into the jugular infusion catheter as a priming dose. Then [$^2H_5$]Phe and [$^2H_2$]Tyr (1.7 and 1.0 mmol/L saline respectively) were continuously infused by a multichannel peristaltic pump (AC-2120, Atto Co. Ltd., Japan) at rates of 3.0 and 1.8 μmol/kg $BW^{0.75}$/h respectively through the same catheter for last 4 h of the study.

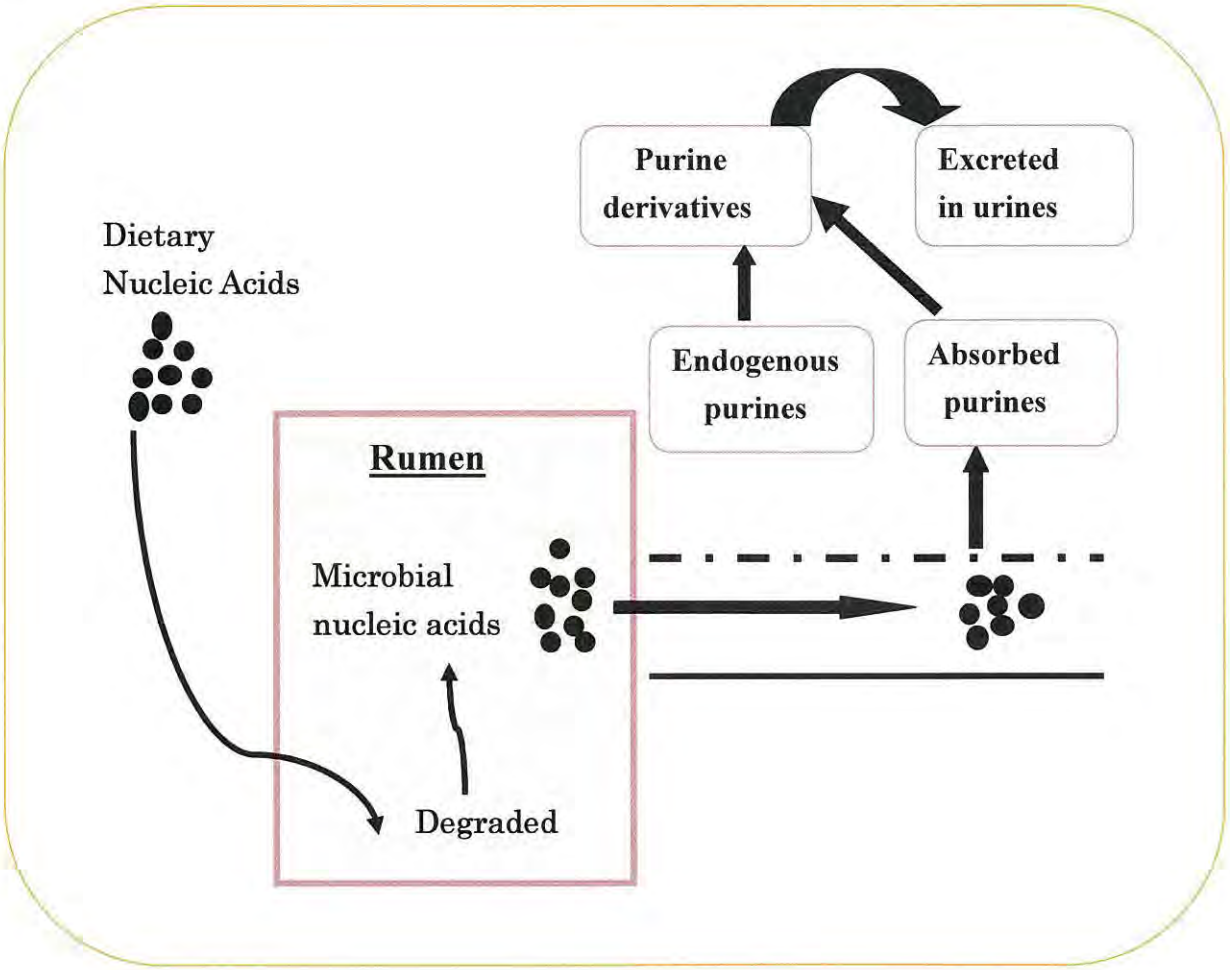

Figure 2.1. Schematic presentation of PD excretion in urine

(Chen and Gomes, 1992).

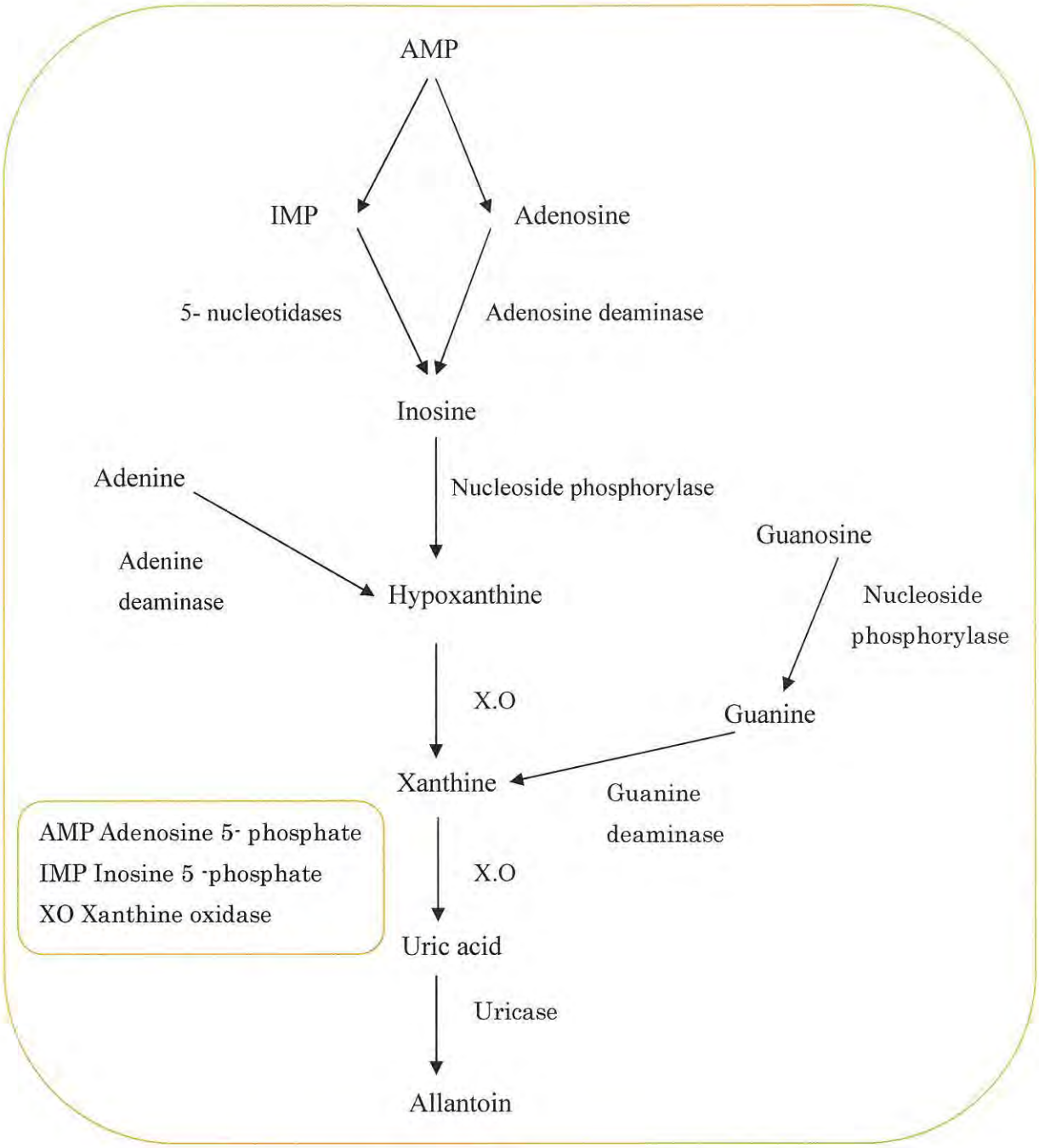

Figure 2.2. Degradation of purine nucleotides and formation of PD (Chen and Gomes, 1992).

Blood samples were taken immediately before (10 mL) and at 30 min intervals (5 mL) over the last 2 h of isotope infusion. The blood samples were transferred into

heparinized tubes containing sodium heparin and stored temporarily in chilled ice until centrifugation. After the end of the experiment, blood samples were centrifuged at 8000 rpm for 10 min at 2℃ and plasma was harvested from blood cells and was stored at -30℃ for later analysis.

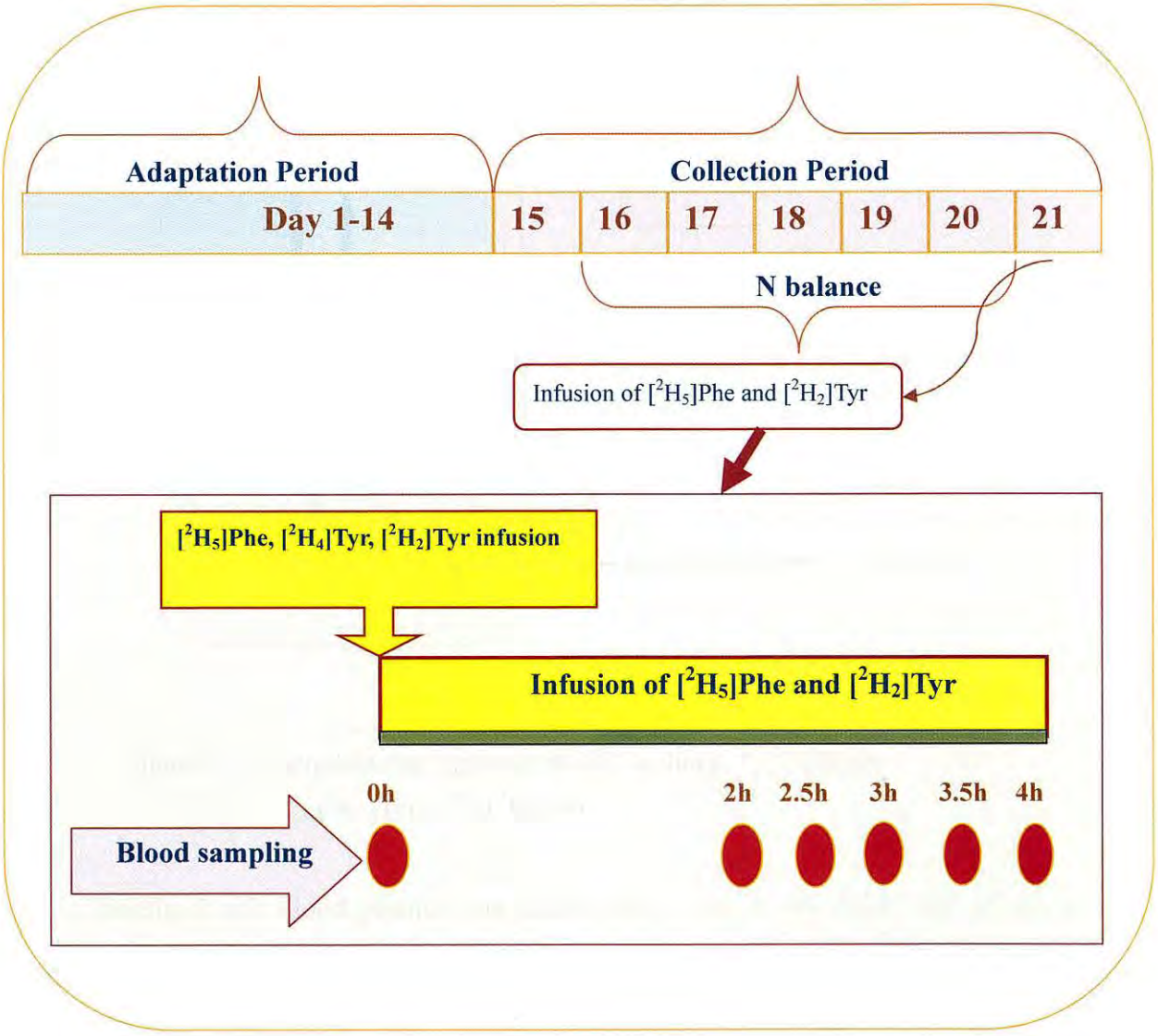

Figure 2.3. Experimental layout and $[^2H_5]$Phe and $[^2H_2]$Tyr infusion method.

Chemical analyses

The concentrations of plasma Phe and Tyr and enrichments of plasma $[^2H_5]$Phe,

[$^2H_4$]Tyr and [$^2H_2$]Tyr were determined using a gas chromatography mass spectrometry (QP-2010, Shimadzu, Japan) by the procedures of Rocchiccioli et al. (1981) and Calder and Smith (1988) as reported by Fujita et al. (2006).

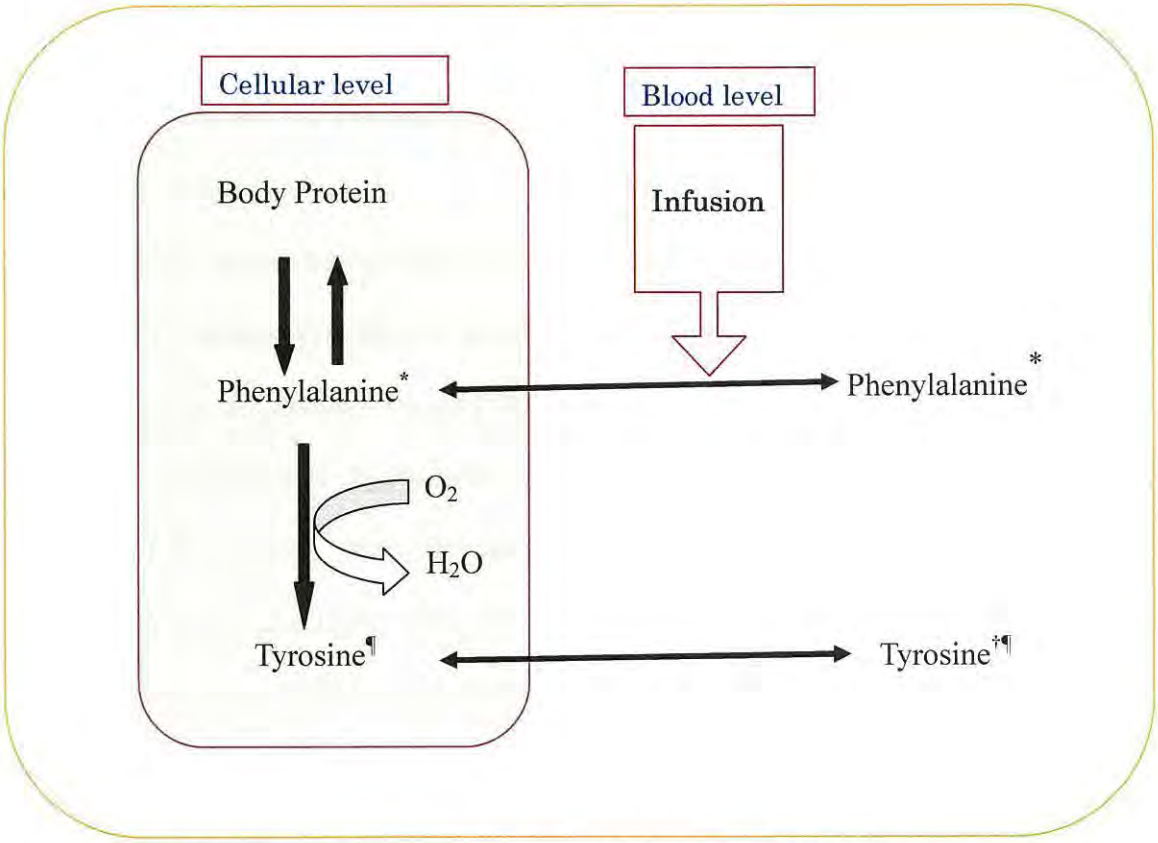

Figure 2.4. Phenylalanine turnover model in sheep. *, [$^2H_5$]Phe;
†, [$^2H_2$]Tyr; ¶, [$^2H_4$]Tyr

Briefly, 1 mL blood plasma was added with 1 mL of 4% SSA, 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 into refrigerator for 30 min. Then, the samples were subjected to centrifuge (0℃, 12000 rpm, 10 min) twice and upper transparent supernatant was poured to column consisted of 0.5 mL of cation exchange resin (Dowex 50 X8 (H^+ form, 200-400 mesh) and washed with 0.5 mL distilled water twice. Then, 1 mL of 4N

NH_4OH added twice and washed finally with 1 mL distilled water. Thereafter, 0.5 mL of the obtaining eluant was transferred into screw capped glass tube and kept into dessicator for drying. After drying, samples were mixed with 25 µL of acetinitrile and MTBSTFA, heated at 80°C for 20 min, and enrichments were measured by gas chromatography mass spectrometry with selection ion monitoring. The ions monitored are: m/z 336 and 341 for Phe and $[^2H_5]$Phe and m/z 466, 468 and 470 for Tyr, $[^2H_2]$Tyr and $[^2H_4]$Tyr respectively.

The PD excretion, namely allantoin, xanthine plus hypoxanthine and uric acid from diluted urine samples were determined by the procedure described by Chen and Gomes (1992) using the spectrophotometer (V-630 BIO, JASCO, Japan; Figure 2.5). With this procedure, xanthine and hypoxanthine were measured together as uric acid after treatment of the urine sample with xanthine oxidase. For allantoin determination, 1 mL of urine and standard samples were taken into 15 mL glass tubes, mixed with 5 mL of distillated water and 1 mL of NaOH (0.5 mol/L). Then, the tubes were placed in the boiling water bath. After boiling for 7 min, tubes were cooled and added with 1 mL of HCl (0.5 mol/L) and 1 mL of phenylhydrazine (0.023 mol/L) and the tubes were boiled again for another 7 min. After boiling, the tubes were dumped into chilled ice for several min and then 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 calculated (mg/L) based on the liner equation. For uric acid, 2.5 mL of urine and standard samples were taken into 10 mL glass tubes and mixed with 1 mL of phosphate buffer (KH_2PO_4, 0.67 mol/L). Two sets of standards 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℃ 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, 1.0 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 standards and samples in duplicate were prepared. In one set 150 μL of phosphate buffer and in other set 150 μL of xanthine oxidase solution were added, mixed properly and incubated in the water bath at 37℃ for 60 min. Then the absorbance was read at 293 nm and xanthine plus hypoxanthine concentration was calculated based on the standard equation.

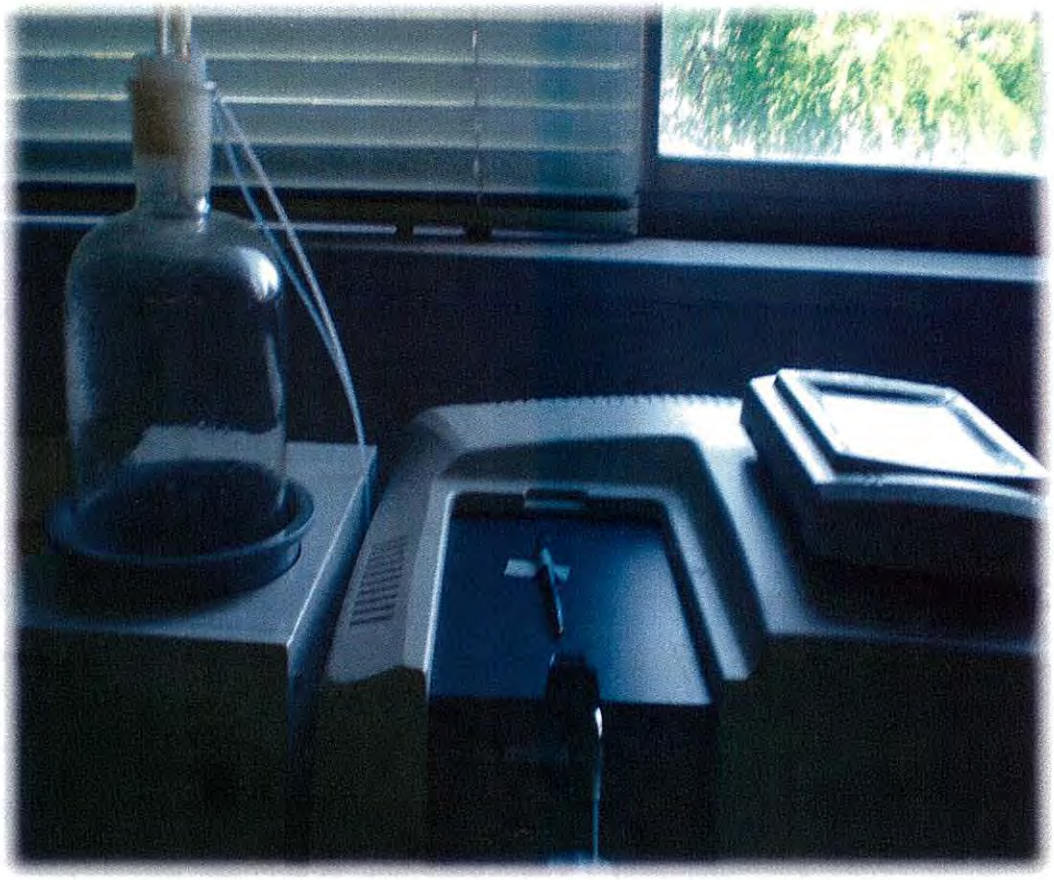

Figure 2.5. Spectrophotometer (V-630 BIO, JASCO, Japan).

Nitrogen contents in diets, faeces and urine and NDF contents in diets were

determined according to the methods stated in Chapter-1. Concentration of plasma glucose was determined enzymatically by the method of Huggett and Nixon (1957). Concentrations of plasma NEFA were also enzymatically determined using the kit (NEFA C, Wako Pure Chemicals Industries Ltd., Japan). Plasma free amino acids, NH_3 and urea concentrations were determined using the automatic amino acid analyser (JLC-500/V, JEOL, Japan) described briefly in Chapter-1.

Calculations

Estimation of MNS:

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

$$Y = 0.84\ X + (0.150\ BW^{0.75}\ e^{-0.25X})$$

where, 0.84 is the recovery of absorbed purines as PD in urine and $0.150BW^{0.75}$ represents the endogenous contribution of purine excretion. The calculation of X from Y based on the equation was made by means of the Newton's iteration process.

Finally MNS was calculated (Chen and Gomes, 1992) as follows:

$$MNS\ (g/d) = 70 \times X\ (mmol/d) / 0.83 \times 0.116 \times 1000$$

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 (Chen, 1989).

Calculation of PheTR, TyrTR and WBPS:

The plasma PheTR and TyrTR were calculated using the following equation by Thompson et al. (1989).

$$TR\ [\mu mol / kg\ BW^{0.75} / h\ ] = I \times (1 / E - 1)$$

where, I is the infusion rate of each stable isotope and E is the respective plasma isotopic enrichments during the steady state conditions. The rate of Phe hydroxylation to Tyr (the rate of Phe conversion to Tyr, PheOX) was calculated as follows:

$$\text{PheOX } (\mu\text{mol / kg BW}^{0.75}\text{/ h}) = \text{TyrTR } (E_{tyr} / E_{phe}) \times (\text{PheTR } / (I_{phe} + \text{PheTR}))$$

where, E_{tyr} and E_{phe}, are the plasma isotopic enrichment of $[^2H_5]$Phe and $[^2H_4]$Tyr, respectively, and I_{phe} is the infusion rate of $[^2H_5]$Phe. The WBPS was derived as follows:

$$\text{WBPS (g/ kg BW}^{0.75}\text{/d)} = (\text{PheTR - PheOX}) / \text{Phe in carcass protein}$$

The Phe concentration in carcass protein was assumed to be 35 g/kg (Harris et al., 1992).

Statistical analysis

All data were analysed using the MIXED procedure of SAS (1996). The fixed effects in the model were diet and period. The random effect was sheep. Results were considered significant at the $P < 0.05$ level, and a tendency was defined as $0.05 \leqq P < 0.10$.

Results:

Nitrogen balance data are presented in Table 2.1. For both treatments, animals were in positive N balance. Nitrogen intake was not statistically different between the diets. Faecal excretion of N tended to be lower ($P = 0.05$) for the GL-diet than Hay-diet. Nitrogen excretion in urine did not differ between the diets. The digestibility and absorption of N were higher ($P < 0.05$) for the GL-diet than Hay-diet. Urinary allantoin excretion was significantly higher ($P = 0.03$) in GL-diet and uric acid and xanthine plus

hypoxanthine excretions did not differ between the diets (Table 2.2). Total PD excretion in the urine and resulted MNS to sheep was also higher (P = 0.04) in GL-diet than Hay-diet.

Table 2.1 Effects of feeding garlic stem and leaf on N absorption, N retention and N digestibility in sheep[a].

Items	Treatment[b]		SEM[c]	*P*-value
	Hay-diet	GL-diet		
N intake (g/kg $BW^{0.75}$/d)	1.39	1.41	0.03	0.29
N in faeces (g/kg $BW^{0.75}$/d)	0.58	0.54	0.01	0.05
N in urine (g/kg $BW^{0.75}$/d)	0.50	0.51	0.02	0.42
N absorption (g/kg $BW^{0.75}$/d)	0.81	0.86	0.02	0.01
N retention (g/kg $BW^{0.75}$/d)	0.32	0.35	0.03	0.42
N digestibility (%)	58	61	1.2	0.02

[a] Values represent means of four sheep.

[b] Hay-diet, mixed hay of orchardgrass and reed canarygrass hay;
GL-diet, hay plus garlic stem and leaf (at a ratio of 9:1).

[c] SEM, standard error of the mean.

Concentrations of plasma free amino acids, NH_3 and urea determined at the pre-infusion period of the isotope dilution method are shown in Table 2.3. The concentrations of plasma valine, isoleucine, leucine and glutamine were significantly higher ($P < 0.05$) in GL-diet than Hay-diet. Plasma glucose concentration did not differ between the dietary treatments and plasma NEFA concentration tended to be lower (P =

0.05) in GL-diet compared with Hay-diet. Plasma NH_3 and urea concentrations did not differ between the diets.

Table 2.2 Effects of feeding garlic stem and leaf on PD excretion and MNS in sheep[a].

Items	Treatment[b]		SEM[c]	*P*-value
	Hay-diet	GL-diet		
Allantoin (mmol/kg $BW^{0.75}$/d)	0.36	0.47	0.03	0.03
Uric acid (mmol/kg $BW^{0.75}$/d)	0.05	0.08	0.01	0.11
Xanthine plus hypoxanthine (mmol/kg $BW^{0.75}$/d)	0.06	0.08	0.01	0.42
Total PD (mmol/kg $BW^{0.75}$/d)	0.48	0.63	0.04	0.04
Total MNS (g/kg $BW^{0.75}$/d)	0.40	0.54	0.03	0.04

[a] Values represent means of four sheep.

[b] Hay-diet, mixed hay of orchardgrass and reed canarygrass hay;
GL-diet, hay plus garlic stem and leaf (at a ratio of 9:1).

[c] SEM, standard error of the mean.

The enrichments of plasma [$^2H_5$]Phe, [$^2H_4$]Tyr and [$^2H_2$]Tyr reached almost plateaus during the latter periods of the isotope dilution method for each treatment (Figure 2.6). The concentrations of plasma Phe and Tyr are also given in Figure 2.7. Kinetics of plasma N metabolism determined by [$^2H_5$]Phe model are presented in Table 2.4. Plasma PheTR determined during the later periods of the isotope dilution method,

Table 2.3 Effects of feeding garlic stem and leaf on plasma free amino acids, NH_3, urea, glucose and NEFA concentrations at the pre-infusion period in sheep[a].

Items	Treatment[b]		SEM[c]	*P*-value
	Hay-diet	GL-diet		
Essential amino acids (μmol/ L)				
Threonine	260	247	16	0.72
Valine	242	272	10	0.04
Methionine	13	13	1	0.43
Isoleucine	85	103	4	<0.01
Leucine	111	139	7	0.01
Phenylalanine	61	77	5	0.06
Histidine	59	47	3	0.07
Lysine	113	120	5	0.12
Non essential amino acids (μmol/ L)				
Serine	131	153	10	0.36
Asparagine	120	113	7	0.38
Glutamic acid	79	90	3	0.09
Glutamine	504	615	26	0.04
Glycine	422	460	24	0.46
Alanine	164	153	5	0.31
Tyrosine	89	94	5	0.33
Tryptophan	65	80	5	0.08
Arginine	151	144	12	0.54
Proline	92	83	6	0.05
Ammonia (μmol/ L)	117	117	2	0.99
Urea (mmol/ L)	3.87	3.72	0.30	0.16
Glucose (mmol/ L)	2.90	2.78	0.11	0.28
NEFA (μEq/ L)	129	112	7	0.05

[a] Values represent means of four sheep.

[b] Hay-diet, mixed hay of orchardgrass and reed canarygrass hay; GL-diet, hay plus garlic stem and leaf (at a ratio of 9:1).

[c] SEM, standard error of the mean.

tended to be greater ($P = 0.06$) in GL-diet and plasma TyrTR did not differ between the diets.

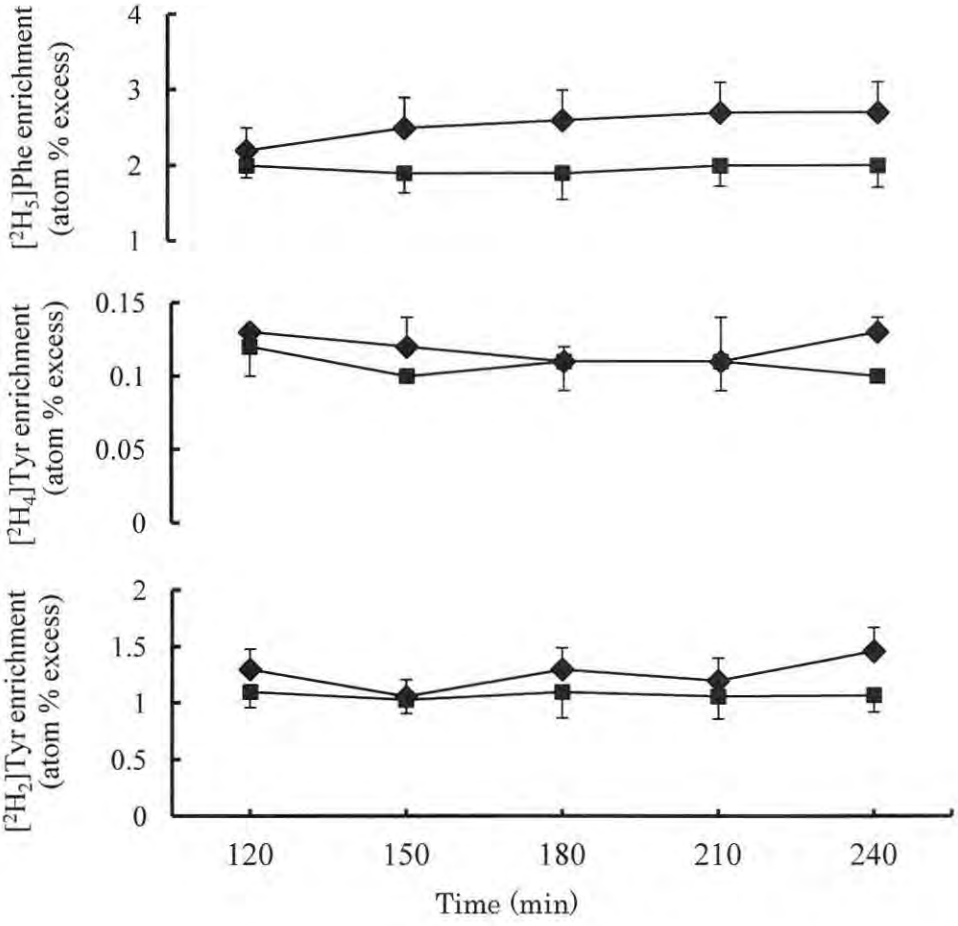

Figure 2.6. The enrichments of Plasma [$^2H_5$]Phe, [$^2H_4$]Tyr and [$^2H_2$]Tyr during the latter period of isotope infusion in sheep (Hay-diet, —◆— and GL-diet, —■—).

Further, PheOX did not differ between the two diets and WBPS tended to be higher ($P = 0.05$) in GL-diet than that of Hay-diet. A positive relationship ($R^2 = 0.58$, $P < 0.05$) was found between MNS and WBPS in the study.

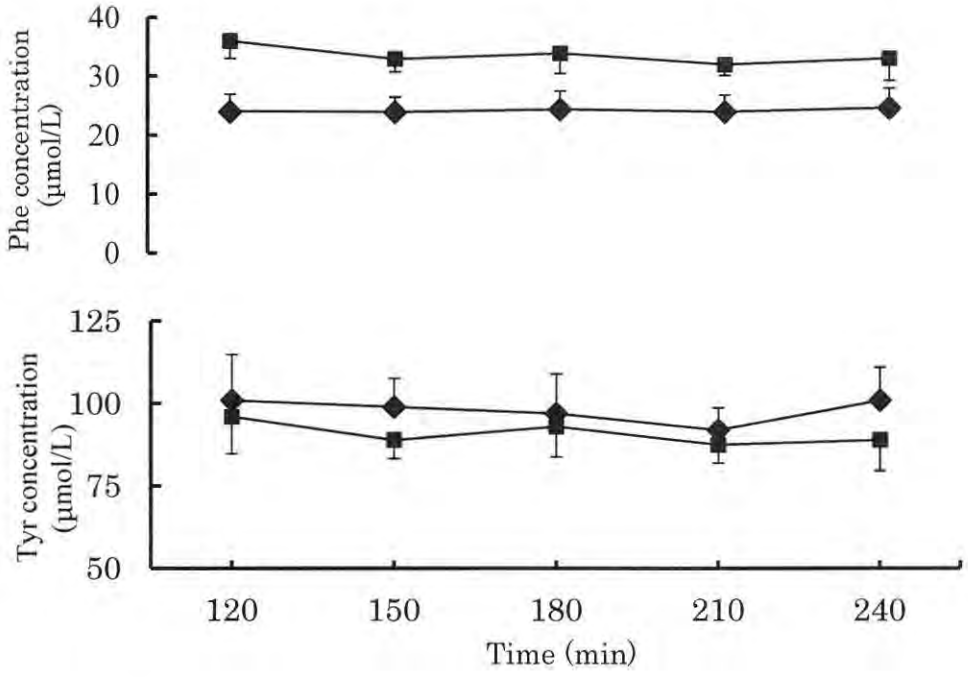

Figure 2.7. The concentrations of plasma Phe and Tyr during the later periods of isotope infusion in sheep (Hay-diet, —◆— and GL-diet, —■—).

Discussion:

In this study, urinary excretions of PD were used to estimate MNS to sheep. Ruminal microbes form the main source of protein to the ruminant. Most ruminant feeds contain a certain amount of nucleic acid and in the rumen dietary nucleic acids are extensively broken down by micro-organisms. Therefore, the nucleic acids arriving at the lower gut is essentially from microbial cells. The purines from the rumen microbes are metabolized and excreted through urine as their end products: allantoin, uric acid and xanthine plus hypoxanthine. Thus, the PD excretion used as an index for the calculation of MNS in ruminants. Limited data in the literature but there is evidence that increasing dietary intake may increase digesta and microbial flow in ruminants (Chen et

al., 1992).

Table 2.4 Effects of garlic stem and leaf feeding on plasma Phe and Tyr concentrations, PheTR and TyrTR, PheOX and WBPS in sheep[a].

Items	Treatment[b]		SEM[c]	*P*-value
	Hay-diet	GL-diet		
Concentrations (μmol/L)				
Phe	24	34	2	0.09
Tyr	100	92	5	0.10
Turnover rate (μmol/kg $BW^{0.75}$/h)				
Phe	91	123	7	0.06
Tyr	111	134	7	0.12
PheOX (μmol/kg $BW^{0.75}$/h)	4.8	7.0	0.8	0.11
WBPS (g/kg $BW^{0.75}$/d)	9.8	13.1	0.8	0.05

[a] Values represent means of four sheep.

[b] Hay-diet, mixed hay of orchardgrass and reed canarygrass hay; GL-diet, hay plus garlic stem and leaf (at a ratio of 9:1).

[c] SEM, standard error of the mean.

In the present study, the higher use of N was in accordance with a previous study by Wanapat et al. (2008) who investigated the effect of garlic powder in cattle and found significantly higher digestibility, absorption and retention of N in supplemented groups compared to control group. In another report, it was found that garlic powder supplementation increased N utilization in finishing pigs (Chen et al., 2008). Cullen et al. (2005) found higher N and organic matter digestibility in pigs offered low levels of

garlic (1g/ kg) in basal diet. These variables can be ascribed to several aspects. It was reported that garlic bioactive components could act as remedy for intestinal disorders, flatulence, worms and respiratory infections and many other ailments because of its potent and widespread effects (Amagase et al., 2001). Such positive effects in the digestive tract might be also responsible for increased N use and MNS to sheep in GL-diet than Hay-diet. Garlic bioactive components might have positive impacts on N use by influencing microbial proteolytic activities of rumen fluid in sheep fed with GL-diet. Garlic bioactive components have antioxidant effects (Prasad et al., 1995; Ide et al., 1997; Amagase, 2006) which might play a role to improve N use in sheep fed GL-diet.

Concentrations of plasma valine, isoleucine, leucine and glutamine were higher in GL-diet which is not consistent with the initial study. It might be due to variations of feed, feeding time and animals in the study. Cheong et al. (2006) also found higher concentrations of some plasma free amino acids in garlic supplemented group than the control in their study. Plasma glucose concentration did not differ between the diets and plasma NEFA concentration tended to be lower in GL-diet which was in accordance with our previous findings (Chapter-1).

There are a number of well established isotope dilution methods using radioactive and stable isotopes to estimate whole body protein metabolism in humans and animals (Wolfe 1984; Marchini et al., 1993; Sano et al., 2004). In our current study, [$^{2}H_{5}$]Phe model was used to determine WBPS in sheep. Of the isotopic tracers methods, [1-^{13}C]Leu is the most widely used method for determining whole body protein synthesis and degradation in human and animals (Krishnamurti and Janssens, 1988; Sano et al., 2008; Al-Mamun et al., 2007), because Leu is either utilized for protein

synthesis or oxidized to CO_2 via α-KIC. For the studies of whole body protein kinetics [$^2H_5$]Phe model proposed by Thompson et al. (1989) was applied mainly for humans and a few experiments have been reported in ruminants (Harris et al., 1992; Fujita et al., 2006; Al-Mamun et al., 2007). For determining WBPS, [$^2H_5$]Phe model is comparable to [1-^{13}C]Leu model (Al-Mamun et al., 2007). In [$^2H_5$]Phe model, WBPS could be measured accurately and quickly as because this method does not require the production and enrichments of expired CO_2. Further, [$^2H_5$]Phe model has some limitations which were clarified by Fujita et al. (2006). The current study demonstrated that GL feeding might have positive effects on plasma PheTR and WBPS in sheep. Until recently, to the best of our knowledge, effects of feeding garlic constituents on amino acids kinetics and WBPS have not been investigated clearly either in humans or in animals. The values of PheTR, TyrTR and WBPS found by our present study were comparable with the previous results (Fujita et al., 2006; Sano et al., 2010). Turnover of plasma amino acids and WBPS increased with increasing dietary intake in ruminants (Harris et al., 1992; Fujita et al., 2006; Sano et al., 2008). Thus, Sano et al. (2008) used different levels of CP intake in heifers and found that LeuTR and WBPS were significantly greater for high CP intake group than for the low and medium CP intake groups. Turnover rates of plasma amino acids and WBPS were also positively correlated with dietary protein and metabolizable protein intake in humans and animals studies (Liu et al., 1995; Motil et al., 1996; Pannemans et al., 1997; Lapierre et al., 2002). Fujita et al. (2006) determined WBPS using the [$^2H_5$]Phe model and reported that WBPS increased in response to increasing energy intake in adult goats. Similarly, Sano et al. (2010) reported an increased WBPS in sheep in high ME group than medium ME group. The current study demonstrated that garlic stem and leaf influenced plasma PheTR and resulting WBPS in

sheep, even though diets were around isoenergetic and isonitogenous. These might be partly attributed due to the effects of plenty of bioactive components of garlic stem and leaf in GL-diet by influencing digestive and metabolic functions in the body. It has been stated that some important immune related blood parameters might be enhanced by garlic constituents (Chen et al., 2008). Garlic constituents have been reported to increase the hormones (LH and testosterone) related to protein anabolism and decrease the hormones (corticosterone) responsible to protein catabolism in rat (Oi et al., 2001). It is also evident that garlic components might have stimulating effects on liver functions which play a significant role in metabolism of nutrients (Amagase, 2006). In my previous findings (Chapter-1), the garlic stem and leaf silage did not affect N metabolism in sheep but in the present study fresh garlic stem and leaf resulted higher N use in sheep. This variation is probably due to the fact that fresh garlic stem and leaf contained higher amount of bioactive components than garlic stem and leaf silage.