EFFECT OF ETHANOL CONTAINED IN APPLE POMACE SILAGE ON POSTPRANDIAL METABOLISM OF SUFFOLK SHEEP

A DISSERTATION SUBMITTED

BY

SHILPI ISLAM

DOCTOR OF PHILOSOPHY

BIOPRODUCTION SCIENCE

THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES

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IN

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SPECIALTY: BIOPRODUCTION SCIENCE

FACULTY OF AGRICULTURE AND LIFE SCIENCES

HIROSAKI UNIVERSITY

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September, 2014

DEDICATED

TO

My devoted husband

Mohammad Nazrul Islam

and

Our beloved son

Shadman Sami (Shanon)

CONTENTS

CHAPTER 1

General Introduction

Efficient utilization of nutritionally rich agro-industrial by-products is a major issue throughout the world because of economic, to improve the feed self-sufficiency rate for the domestically raised animal and environmental concerns. Agro-industrial by-products are residues obtained after processing of fruits, vegetable and crops which could profitable be used are available locally but are not fully exploited for the feeding of livestock. They may include citrus pulp, tomato pomace, grape pomace, apple pomace, etc.

Apple pomace

Apple pomace (AP) is the primary by-product of apple processing industry, is a rich source of many nutrients including large amounts of soluble carbohydrate, mineral, and organic acids but poor in crude protein (CP). Approximately, 160,000 tons of AP is generated annually in Japan (Takahashi and Mori, 2006). It's could be used for ruminant feed stuff (Alibes et al., 1984; Gasa et al., 1992; Taasoli and Kafilzadeh, 2008). The storage of this by-product is difficult due to its high moisture content which above 700 g/kg (Kennedy et al., 1999). After leaving the factory, it is therefore necessary to utilize immediately or to conserve it by a process of ensiling or dehydration.

Apple pomace silage

Apple pomace silage (APS) is a fermented feed for ruminants that prepared with AP and other ingredients to adjust the moisture and CP. Fermented APS has a considerable amount of moisture, higher amount of ethanol, higher amount of lactic acid, lower pH and no butyric acid and produces good quality of silage which is palatable (Alibes et al., 1984; Pirmohammadi, et al., 2006). Apple pomace supplemented with protein source has similar feeding value to grass silage for

wintering beef cattle (Burris and Priod, 1957; Fontenot et al., 1977; Bovard et al., 1977; Oltjen et al., 1977).

Alcohols are normal constituents of silage and silage based rations at various concentrations, depending on the fermentation profile (Kristensen, et al., 2010). Moreover, ensiling apple pomace results in the conversion of most of the water-soluble carbohydrates into fermentation end-products (McDonald et al., 1991). Ethanol is the main fermentation end-product in apple pomace silages (Alibes et al., 1984). Ethanol contents of several silages have been reported to be 12 - 15 g/kg DM for corn silages (McDonald et al., 1991; Raun and Kristensen, 2010), 8 - 14 g/kg DM for grass silages (Lawrence et al., 2011), 10 - 23 g/kg DM for ensiled TMR silages (Cummins et al., 2009), 54 g/kg DM for sugarcane silage (Daniel et al., 2013) and 173 g/kg DM for APS (Alibes et al., 1984). Therefore, most of ruminants are adapted to some ethanol intake. For high ethanol content, APS can be used only in limited amounts (Alibes et al., 1984).

In ruminants, contrary to man and monogastric animals, a significant proportion of any ingested ethanol can be transformed into short chain fatty acids by rumen bacteria (Pradhan and Hemken, 1970; Czerkawski and Breckenridge, 1972; Durix et al., 1991) and is also immediately absorbed across rumen epithelium (Veresegyházy et al., 2003). Ethanol can contribute up to 10% of the energy requirements but excess alcohol intake may result in increased alcohol load into the liver (Jean-Blain et al., 1992) and followed by hours of elevated peripheral concentrations of alcohol (Kristensen et al., 2007). Dietary alcohols are associated with the changes in ruminal fermentation pattern such as increased ruminal proportions of acetate (Rumsy, 1978; Raun and Kristensen, 2011), valerate and caproate (Chalupa et al., 1964; Durix et al., 1991). A similar change in ruminal VFA proportion has been observed after feeding of APS (Fang, 2009).

Ethanol metabolism and its influence on gluconeogenesis in ruminants

On the basis of ruminant nutrition, ethanol metabolism related into at least 4 serially arranged metabolic entities such as silage, rumen, ruminal epithelium and liver. Ethanol can be synthesized in the rumen by fungi (Teunissen et al., 1992) and bacteria

(Lauková and Marounek, 1992), and remove from the rumen by microbial metabolism and absorption (Jean-Blain et al., 1992). Dietary ethanol partly metabolized by ruminal microbes and excess amount absorbed across the rumen epithelium. Ethanol goes to liver for metabolism and increases alcohol load on the liver. Ethanol is eliminated from the body mainly by oxidation. The liver parenchymal cell (hepatocyte) contains alcohol dehydrogenase and aldehyde dehydrogenase enzymes which oxidize ethanol into acetate. This pathway generates an overabundance of cytosolic NADH (nicotinamide adenine dinucleotide) and elevated the $NADH/NAD^+$ ratio (Kerbs, 1968). The consequence of the shift in the redox state is commonly held to be the mechanism responsible for the inhibition of gluconeogenesis (Kerbs, 1968; Kerbs, et al., 1969). Recently, Kondo et al. (2011) found that plasma glucose concentration was decreased with APS ethanol ingestion in sheep and Brand-Miller et al. (2007) also reported that alcoholic beverage consumption lowered postprandial glycaemia in human which may be caused by ethanol's actions of suppression of hepatic gluconeogenesis and glucose output. Under such conditions of suppressed gluconeogenesis, gluconeogenic precursors including lactate in the circulation would be elevated. Concomitant problems of hypoglycemia and hyperlactatemia might lead to hyperketonemia and then end up in ketosis (Fig 1.1).

Hepatic glucose is the major determinant of plasma glucose concentration (Fèry, 1994) regardless of the nutritional state. The inhibitory effect of ethanol upon hepatic gluconeogenesis resulting in a lower glucose output, which could lead to ethanol-induce hypoglycemia especially in glycogen-depleted individuals. Because glycogen, the primary storage form of glucose in animal tissues and it occurs mainly in the liver, plays a central role in systemic glucose homeostasis. Glycogen in muscle provides a reservoir of glucose for muscle activity, whereas liver glycogen is involved in the regulation of blood glucose levels by storage of excess glucose after meals and the subsequent release of glucose between meals. Thus dietary ethanol may have an important role for glycogen synthesis.

On the other hand, Vrzalová and Zelenka (2001) reported that the fermentation of readily fermentable energy in the rumen will result in enhanced production of volatile fatty acids and lactate. These changes in end products of rumen fermentation would consequently influence blood glucose metabolism and endocrine (Sowinska et al., 2001; Sano and Fujita, 2006), possibly through the regulation of gene expression of key enzymes for gluconeogenesis in liver (Hesketh et al., 1998). Gluconeogenesis in ruminants occurs mainly in the liver with the kidney accounting for a maximum of about 15%. The major hepatic substrate cycles in gluconeogenesis pathway in liver shown in Figure 1.2.

Objectives of the study

The main purpose of the study was to develop a nutritionally balanced APS for ruminants that prepared with AP and other ingredients which have similar TDN content to a commercial concentrate and to investigate the effect of feeding APS that rich in ethanol. For this a series experiment was undertaken with the following objectives:

1. To investigate the effect of APS ethanol on plasma ethanol, lactate, β-hydroxybutyrate (BHBA), insulin, metabolites, and the liver enzyme activity of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) in Suffolk sheep by different sampling windows.
2. To evaluate the fermentation characteristics and nutritive value of alcoholic APS.
3. To investigate the effect of APS ethanol intake on liver glycogen content.
4. To investigate the influence of APS ethanol on gene expression of some regulatory gluconeogenic enzymes of the intermediary ethanol metabolism; pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1, 6-bisphosphatase (FBP1), glucose-6-phosohatase (G6Pase) and Glycogen synthase (GS).

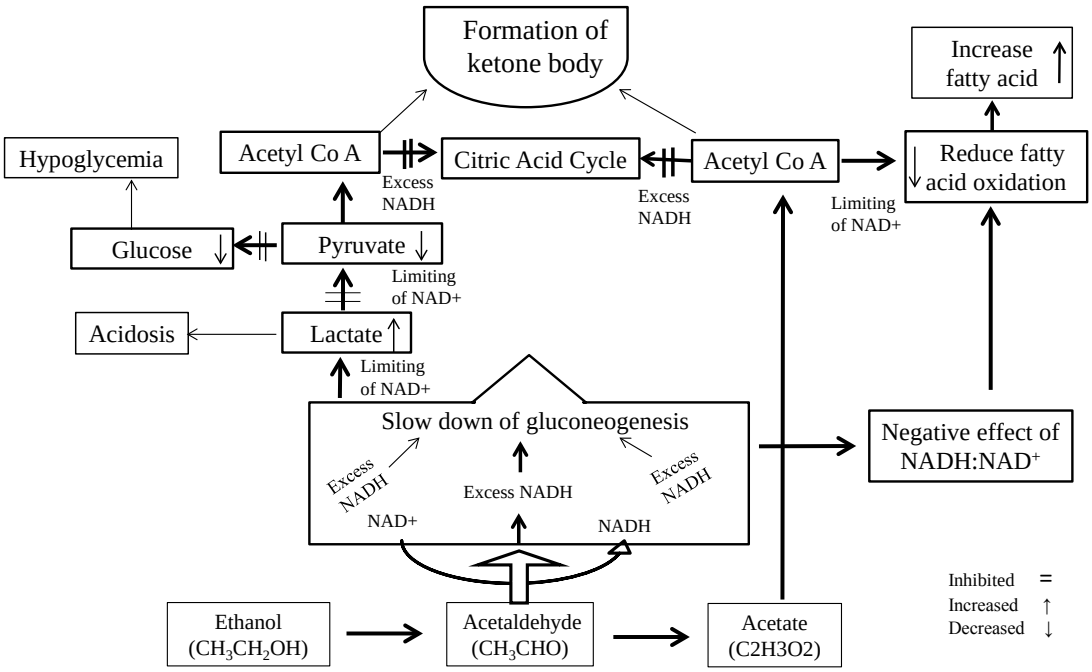

Figure 1.1: Scheme diagram of Ethanol metabolism effects.

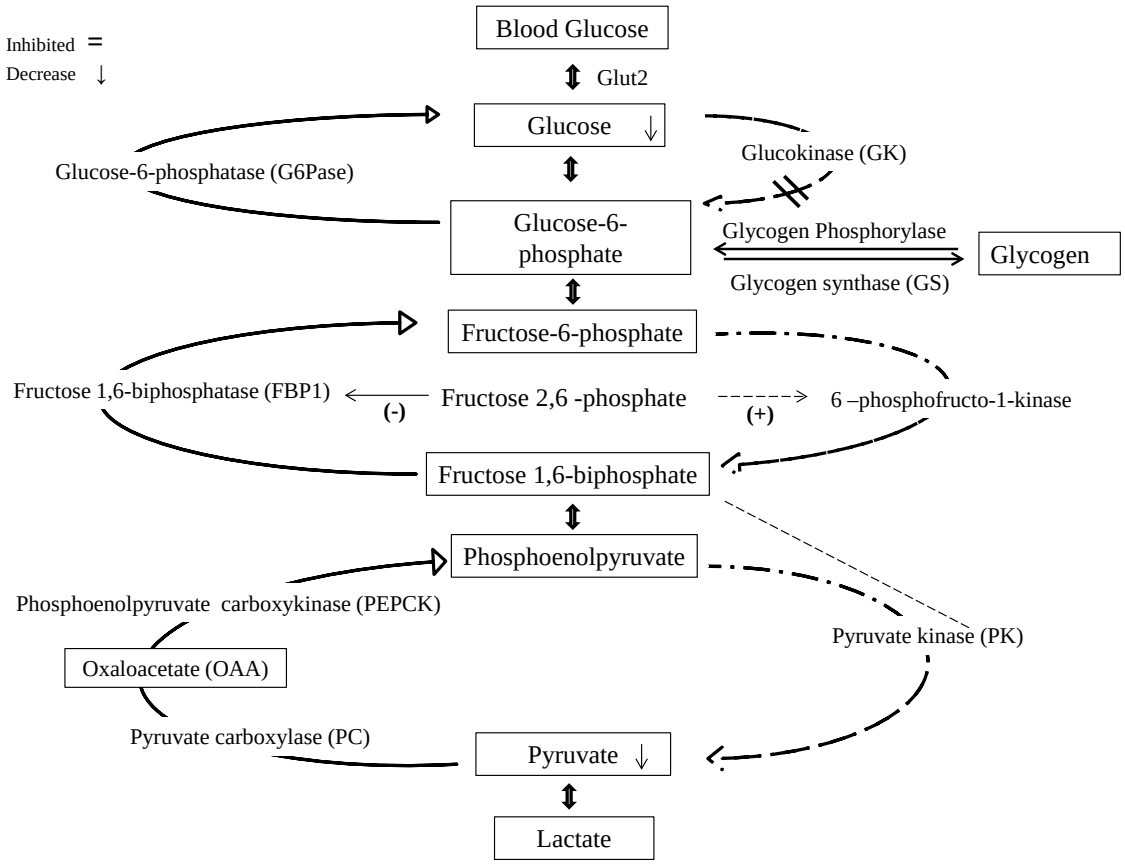

Figure 1.2: Substrate cycles in the gluconeogenesis/glycolysis pathways that are involved in the regulation of glucose production by the liver. The line arrow circle show the gluconeogenesis pathway and the dashed line arrow show the glycolysis pathway (Adapted from Pilkis and Granner, 1992).

CHAPTER 2

Effect of feeding apple pomace silage ethanol on response of blood parameters in Suffolk ewes

CHAPTER 2.1

Effect of feeding rice straw apple pomace silage on response of blood parameters in Suffolk ewes

INTRODUCTION

Apple pomace (AP), a by-product of the apple processing industry which could be used for ruminant as a good energy source but poor in protein (Alibes et al., 1984; Gasa et al., 1992). Apple pomace contains high amounts of moisture, fermentable carbohydrate and organic acids which are responsible for prominent production of ethanol during ensiling. Ethanol is a normal constituent of ensiled or fermented feeds at various concentrations, depending on ingredients of the materials and fermentation pattern. Major constrain of fresh AP and apple pomace silage (APS) for ruminants are low protein and high ethanol content (fresh AP and APS were 50.5 and 173.3 g kg^{-1} DM, respectively) and APS can be used only in limited amounts (Alibes et al., 1984). However, some studies were undertaken to utilize the AP with urea supplementation for balance the low protein content (Rumsey, 1978; Nikolić and Jovanović., 1986). Urea may not be an appropriate source of nitrogen in rations containing high proportions of AP which is the problem of birth weight, deformities and mortality in calves from beef cows (Fontenot, et al., 1977). When ration included straw (20-25%) then those problem do not occur (Rumsey et al., 1979). Recently, Kondo et al. (2010) also reported that APS contains a considerable amount of ethanol (37.2 - 94.9 g kg^{-1} DM).

However, some studies were undertaken about the dietary alcohols are associated with the changes in ruminal fermentations pattern (Rumsy, 1978; Raun and Kristensen, 2011, Chalupa et al., 1964; Durix et al., 1991, Fang, 2009) and peripheral blood (Jean-Blain et al., 1992; Kristensen et al., 2007) but information on the impact of APS ethanol on blood biochemistry is limited.

Recently, Kondo et al. (2011) found that plasma glucose concentration was decreased with APS ethanol ingestion in sheep and Brand-Miller et al. (2007) also reported that alcoholic beverage consumption lowered postprandial glycaemia in human which may be caused by ethanol's actions of suppression of hepatic gluconeogenesis and glucose output. These early studies confirm the inhibitory effect of ethanol upon resulting in lower glucose output, and under such conditions gluconeogenic precursors including lactate in the circulation would be elevated which could lead to alcohol-induce hyperketonemia .

Therefore, the current study was undertaken to investigate the effect of feeding RS-APS that is rich in ethanol on blood parameters in Suffolk ewes.

MATERIALS AND METHODS

1. Animals, experimental design and management

All procedures involving animals in this study were approved by the Institutional Animal Care and Use Committee at Hirosaki University (A08023).

Four Suffolk shorn ewes, aged 7 months on average, weighing 39 ± 4 kg, were used in this experiment to study feeding effect of alcoholic fermented rice straw containing APS (RS-APS) on blood parameters. The experiment was performed using crossover design over two 35-day periods including of initial 7 days for dietary adaptation. In this experiment, sheep were allocated to two dietary treatments; alfalfa hay cube and either RS-APS or a commercial concentrate (CP 15.5% and TDN 70%; control) at a ratio of 30:70 on TDN basis. During the first period two sheep were fed on control diet and then fed on RS-APS diet during the second period. The other two ewes received the treatment diets in reverse order. Daily allowance was offered as TDN requirement for maintenance and daily 100g gain (Ministry of Agriculture, Forestry and Fisheries, 1996) at 10:00 and 18:00 in 2 equal meals. The required amount was adjusted weekly based on their BW. Ewes were housed in individual pens with sawdust bedding in an indoor animal room and had free access to a trace mineralized salt block and water throughout the experiment.

2. Apple pomace silage preparation

Fresh AP was obtained from an apple juice factory of the Farm village industry federation of Aomori prefectural agricultural cooperatives at Hirosaki, Japan. The RS-APS was prepared with fresh AP, soybean meal, corn, wheat bran and rice straw to balance TDN content similar to a commercial concentrate (Table 2.1.1). For ensiling RS-APS, fresh AP within 2 days of production was used. The AP and all other ingredients were mixed thoroughly by a mechanical mixer and stuffed into plastic container, pressed sufficient to fill properly, topped with air-tight cover and ensiled for at least 2 months before use in the feeding experiments.

3. Feed samples and analyses

One hundred grams of AP or RS-APS sample was extracted with 300 g of distilled water at 4 °C with occasional gentle swirling. After 18 hours of extraction, the aqueous solution was strained through four layers of gauze and further filtered through a filter paper (Quantitative ashless 5A type, Advantec, Japan). The pH was measured immediately by using a digital pH meter (KR5E, AS-PRO, Japan) and then extracted solution was stored at -30 °C for analysis on volatile basic nitrogen (VBN), organic acids and ethanol. The VBN content was determined as described by Conway (1962). Ethanol was determined by a specific gravity meter (DA-310, Kyoto Electronics, Japan) after direct distillation of the extracted sample and organic acids were analyzed by a bromothymol blue post-column method using HPLC (D-2000 HSM, Hitachi, Japan). All feed samples were dried in a forced-air oven at 60 °C for 48 hours, ground through a 1 mm screen using a Willey mill and kept in a plastic airtight container for chemical analyses. Dry matter (DM), crude protein (CP), ether extract (EE), crude ash and crude fiber (CF) contents were determined according to the methods of Association of Official Analytical Chemists (1990). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to the procedures of Van Soest et al. (1991). To determine the NDF, 20 ml distilled water was added with 1 g of ground sample and heat to boiling, cooled at room temperature, 20 ml α-amylase (015-03731,

Wako, Japan; 1 mg heat-stable α-amylase: 20 ml phosphate buffer) solution was added, shake and incubate at 40 °C for overnight. After 16 hours of incubation, the solution was filtered through the pre-weighed filter paper (Quantitative ashless 5A type, Advantec, Japan). Filtered residue were transfer in the beaker and added 100 ml of neutral detergent solution for each sample, heat to boiling and refluxed for 1 hour from the onset of boiling. Filtered residues were washed with boiling water and acetone and then ashed in a muffle furnace for 2 hours at 600 °C. ADF assayed procedure was similar except incubation with α-amylase buffer solution and acid detergent solution was used instead of neutral detergent solution. Chemical composition of feed ingredients used in the RS-APS is presented in Table 2.1.2. The pH value, organic acids and other fermented products of fresh AP and RS-APS are presented in Table 2.1.3. Chemical compositions of experimental feeds are presented in Table 2.1.4.

4. Blood samples collection and analytical methods

On the last day of each 35 day period, series of blood samples were obtained by jugular venipuncture at pre-feeding (0) and at 0.5, 1, 2, 4 and 8 hours after the morning feed. The blood was collected into two 7 mL evacuated tubes containing sodium heparin as an anticoagulant, chilled on ice and centrifuged (3000 × g for 15 min at 4 °C) to harvest plasma sample. Plasma was aliquotted and stored at -30 °C until analyzed for ethanol, lactate, β-hydroxybutyrate (BHBA), insulin and plasma metabolites [glucose, non-esterified fatty acids (NEFA), plasma urea nitrogen (UN), total cholesterol (T-Cho), free cholesterol (F-Cho), high density lipoprotein cholesterol (HDL-Cho), phospholipid and triglyceride]. Another plasma aliquot was stored at -80 °C until analysed for the ethanol-induced liver injury biochemical marker of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT). Plasma ethanol concentration was measured by UV method using an enzymatic F-kit ethanol kit (R-biopharma, Germany). Plasma lactate and BHBA were determined with enzymatic colorimetric methods using medical test instruments, Lactate Pro 2 (Arkray, Japan) and Precision Xceed Pro (Abbott, Japan), respectively. Plasma insulin concentration was

assayed by a sandwich ELISA technique with Mercodia ovine insulin ELISA kit (Mercodia, Sweden). All plasma metabolites were determined with the enzymatic colorimetric method using Wako commercial kits (Wako pure Chemicals, Japan). Glucose was determined by using Glucose CII-test Wako, NEFA was determined by using NEFA C-test Wako, plasma UN was determined by using UN B-test Wako, T-Cho was determined by using Cholesterol E-test Wako, F-Cho was determined by using Free Cholesterol E-test Wako, HDL-Cho was determined by using HDL-Cholesterol E test Wako, phospholipid was determined by using Phospholipid C-test Wako and triglyceride was determined by using Triglyceride E-test Wako. Plasma GOT and GPT activities were determined with an enzymatic colorimetric method using GOT.GPT CII-test Wako (Wako pure Chemicals, Japan). The area upper or under the curve (AUC) for 2 and 8 hours after feeding of plasma parameters was calculated as an indicative of the responses to feeding in each parameters.

5. Calculation

Hemicellulose was calculated as the difference between the NDF and ADF. Non-fibrous carbohydrate (NFC) was calculated by using the equation of NRC (2001): NFC = (100- CP - EE - NDF - Ash). Average body weight gain (BWG) and average TDN intake were calculated for each experimental period. Feed conversion ratio (FCR) was calculated as a ratio of TDN intake to BWG. Ethanol intake was calculated from RS-APS intake and ethanol contents of the RS-APS.

6. Statistical analysis

Performance traits data were analyzed using the general linear model (GLM) procedure of SAS (2003). Sheep, period and treatment were included in the model. Treatment means were separated using a PDIFF option, protected by overall F-test. Difference was considered statistically significant at $P < 0.05$ and considered a tendency toward significance at $0.05 \leq P < 0.10$. Serial data for blood parameters were analyzed with repeated measures ANOVA using GLM procedure of SAS. If the

treatment effect or treatment × time interaction was significant, treatment means within each sampling time were separated by least significant difference procedure. Relationship among the AUC responses for plasma measurements was analyzed by Pearson's correlation coefficient.

RESULTS

1. Performance traits

No health problems were observed in any of ewes throughout the experimental periods. Lsmeans for performance traits of the ewes are presented in Table 2.1.5. No difference was detected in initial and final BWs between the treatments. However, the daily average BWG was lower ($P < 0.05$) in the RS-APS than control group. Average TDN intake did not differ between treatments. The FCR for control was tended to be better ($P = 0.06$) than for RS-APS treatment. Ewes on RS-APS treatment received an average of 22.8 g/d of ethanol whereas ewes on control were assumed to receive any of alcohol.

2. Blood parameters

Time course changes of plasma ethanol, lactate and BHBA are illustrated in Figure 2.1.1. After ingestion of RS-APS, plasma concentrations of ethanol, lactate and BHBA were increased ($P < 0.01$, $P < 0.05$ and $P < 0.05$; respectively) and peaked at 2, 1 and 4 hours after feeding, respectively. These elevated concentrations for RS-APS treatment were eventually returned to the pre-feeding values at 8 hours after feeding.

Time course changes of plasma insulin and glucose are illustrated in Figure 2.1.2. Plasma insulin level in the RS-APS group was tended to increase ($P < 0.1$) at 1 hour after feeding and declined rapidly until 2 hours to the pre-feeding level which is similar to that in control group. Plasma glucose concentrations for both treatments declined over the first 2 hours after feeding ($P < 0.01$) and returned to the previous values at 8 hours after the morning feed, however no significant treatment difference was detected

although the RS-APS group showed somewhat large magnitude of postprandial hypoglycemia.

Time course changes of plasma T-Cho, F-Cho and HDL-Cho are illustrated in Figure 2.1.3. Plasma T-Cho concentration was higher for RS-APS treatment however no significant effect was detected between the RS-APS and control group. After ingestion of RS-APS, plasma concentrations of F-Cho and HDL-Cho were elevated ($P < 0.05$ and $P < 0.01$; respectively) and peaked at 1 hour after feeding for both.

Time course changes of plasma triglyceride and phospholipid are illustrated in Figure 2.1.4. After feeding of RS-APS, plasma triglyceride concentration was temporary decreased at 0.5 hour after feeding and peaked at 4 hours however no significant treatment effect was detected between the treatments. Plasma phospholipid concentration was tended ($P < 0.1$) to increase in RS-APS treatment and higher concentration was observed at 8 hours after the morning feed.

Time course changes of plasma UN and NEFA are illustrated in Figure 2.1.5. After feeding of RS-APS, plasma UN concentration was decreased ($P < 0.05$). Plasma NEFA concentrations for both treatments were declined ($P < 0.01$) over the first 2 to 4 hours and returned to the previous values at 8 hours after the morning feed however there was no significant treatment difference.

Time course changes for the enzyme activity of plasma GOT and GPT are illustrated in Figure 2.1.6. No significant treatment difference was detected for the plasma enzyme activity of GOT and GPT.

3. Response of area upper or under curve (AUC)

The AUC of plasma parameters for 8 hours sampling windows are represent in Table 2.1.6. The AUC of plasma ethanol was greater ($P < 0.05$) for RS-APS treatment than that of control (Figure 2.1.7A). The AUC of plasma BHBA in the RS-APS treatment was also greater ($P < 0.05$) than that of control (Figure 2.1.7B). The AUC of plasma lactate was tended to grater in RS-APS treatment than that of control treatment. Plasma phospholipid AUC was tended to smaller in RS-APS treatment than that of

control. No significant differences were observed between the RS-APS and control treatments for the AUC of plasma insulin, glucose, T-Cho, F-Cho, HDL-Cho, TG, plasma UN, NEFA, GOT and GPT.

4. Correlations among AUC responses of plasma measurements

The correlation coefficients among AUCs of plasma ethanol, lactate, BHBA, insulin, plasma metabolites, GOT and GPT during 2 hours after feeding are presented in Table 2.1.7. The AUC of BHBA was positively correlated with those of ethanol ($P < 0.01$) and lactate ($P < 0.05$). The insulin AUC was positively correlated ($P < 0.01$) with AUCs of ethanol and BHBA. The AUC of plasma BHBA was also positively correlated ($P < 0.05$) with the AUC of GPT. No significant relationship was observed for others plasma parameters.

DISCUSSION

Fresh AP contains considerable amount of soluble sugars which can be an excellent substrate for ethanol fermentation (Hang et al., 1981). In fact in the present study, negligible ethanol content was observed in fresh AP and after 2 month of ensiling, fermented RS-APS contained a considerable amount of ethanol which blended with other ingredients to improve nutritional balance of fresh AP. Alibes et al. (1984) reported the higher amount of ethanol (111.6 g kg^{-1} DM) for apple pomace-straw silage (34.7% straw on DM basis) than that in this study. The RS-APS contained some 2 - 3 times greater ethanol content compared to corn, grass and TMR silages (McDonald et al., 1991; Raun and Kristensen, 2010; Lawrence et al., 2011; Cummins et al., 2009).

A slight but significant reduction in average BWG and a tendency for increased FCR was observed in the RS-APS than control treatment. On the other hand, improved ADG and FCR by feeding of both ensiled and dried AP was reported in finishing lambs (Taasoli and Kafilzadeh, 2008). The RS-APS used in the present study had lower CP and higher fiber contents compared to the concentrate. We have formulated RS-APS blend to have similar energy content to the concentrate, but did not further fortified CP

content of RS-APS because its estimated CP value was sufficient to meet requirement of growing ewe lambs (Ministry of Agriculture, Forestry and Fisheries, 1996). In this study, ewes were provided sufficient energy from concentrate or APS and grew faster than the targeted daily gain of 100 g. Under such energy-sufficient condition, increased CP level over requirement may have stimulated growth of ewes. Therefore, ingested dose of ethanol (~23 g/d) from RS-APS in this study would not cause any harmful influence on performance of growing ewes.

The plasma ethanol, lactate and BHBA concentrations were elevated after feeding of RS-APS and returned to pre-feeding levels until 8 hours of morning feed. This study is the first to report simultaneous postprandial increases in plasma ethanol, lactate and BHBA levels by feeding alcoholic fermented APS. Kristensen et al. (2007) also observed increases in plasma concentrations of ethanol, lactate and BHBA after feeding of corn silages which contained a measurable amount of ethanol (14.2 g/kg DM), however they concluded that typical amounts of alcohols in corn silage do not interfere with splanchnic metabolism of major metabolites and do not saturate hepatic pathways for alcohol metabolism. As Alibes et al. (1984) cautioned against overfeeding of alcoholic fermented APS, ethanol content of RS-APS used in the present study was more than at least twice as much as corn silage (McDonald et al., 1991; Kristensen et al., 2007; Raun and Kristensen, 2010). Nonetheless all of the blood parameters determined in this study returned to pre-feeding levels until 8 hours after morning feed and none of clinical signs of health problem was observed. According to the results of kinetic study of infused ethanol in sheep, Jean-Blain et al. (1992) assumed that daily ethanol intake ranging from 0.2 to 1 g/kg BW can be metabolized by rumen microflora and enzymatic system of the host and plasma ethanol level remains below 0.25 g/L. These estimates are correspond very well to the fact that ewes consumed 0.55 g/kg BW of ethanol and the highest plasma ethanol level was 0.2 g/L observed in the present study. Heitmann et al. (1987) suggested that ketosis in pregnant sheep and lactating cows are prevented by BHBA stimulation of pancreatic insulin production, and this

may explain tendency for temporary increased plasma insulin level and positive correlation between the AUCs of plasma BHBA and insulin levels in this experiment.

It is established that ethanol inhibit hepatic gluconeogenesis from lactate, glycerol, some amino acids and other substrates (Krebs et al., 1969) and thus cause postprandial hypoglycemia in sheep fed on APS diets (Kondo et al., 2010; 2011) and reduced postprandial glycaemia in young adult human subjects consumed alcoholic beverages with a meal (Brand-Miller et al., 2007). Although we failed to demonstrate significant decrease in plasma glucose concentrations in the RS-APS than control treatment, however, elevated plasma lactate and BHBA levels should be an indication of suppressed gluconeogenesis. The lower CP containing RS-APS treatment largely reflected in lower plasma UN level. It can be assumed that insufficient protein was delivered to the cells and influence the lower plasma UN. Owens et al. (2009) and Walsh et al. (2008) also observed that lower plasma urea level seems to be a greater reflection of the N intake. RS-APS ethanol may have a little effect on postprandial cholesterol concentrations. Holtenius and Holtenius (1996) reported that primary factor for the ketotic state is the high demand for glucose and insufficient gluconeogenesis, which is compensated by increased ketogenesis, there are very few prerequisites for lipid synthesis in the liver cell and small risks for fat accumulation of ruminants. Accumulation of fat in the liver can be alleviated by secreting lipids into the blood steam. The negative effects of NADH:NAD^+ on hepatic gluconeogenesis, fatty acid oxidation is reduced. Accompanying with the combination of higher dietary fat and ethanol has a synergistic effect in tended to increase plasma lipid concentration because ethanol suppresses the clearance of intestinally derived chylomicrons. Alcoholic beverage consumption is associated of greater HDL-Cho in humans which may cause by ethanol (Henk, et al., 1998; Frohlich, 1996; Minna, et al., 2003; Van der Gaag et al, 2001). In the present study, we also found a higher concentration of HDL-Cho in RS-APS treatment. Lipoproteins are modified by acetaldehyde, ethanol metabolism effect or presence of alcoholic beverage antioxidants which influences the lipoprotein alterations (Frohlich, J.J., 1996). So, APS ethanol intake impels plasma cholesterol

esterification. We cannot conclude whether and/or how, higher plasma HDL-Cho concentration of APS is beneficial for the health of sheep. Well-fed animals like growing ewes in the present study would have sufficient precursors for ketogenesis. Therefore once ingested ethanol suppressed gluconeogenesis, lactate would become redundant in gluconeogenic pathway and be accumulated and BHBA would also be generated from fatty acids in well-fed ruminant animals. Consistent positive correlations between the AUCs of plasma lactate and BHBA concentrations in this study are strongly supporting this notion. However, we failed to detect ethanol induce liver injury biomarker GOT and GPT toxic effect. Although all of the responses observed in blood parameters are transient in this study and no health problems were detected, need for prolonged feeding trial to assess safety and productivity of the use of APS is clearly warranted.

In conclusion, according to the consistent increase in plasma ethanol and BHBA after feeding of alcoholic-fermented RS-APS, increased lipid profiles for RS-APS group and positive correlation of postprandial BHBA with lactate and ethanol, we concluded that APS ethanol consumption induce postprandial transient hyperketonemia through suppression of hepatic gluconeogenesis.

SUMMARY

This experiment was conducted to investigate the effect of feeding RS-APS ethanol on response of blood parameters in Suffolk ewes. Four ewes were fed alfalfa hay cube and either rice straw APS (RS-APS, 33.4 g of ethanol/kg DM) or concentrate (control) in a 2 × 2 crossover design over two 35-day periods. Alfalfa hay cube and either the concentrate or RS-APS were provided at a ratio of 30:70 of TDN requirement for maintenance and daily 100g gain. On the last day of each 35 days period, blood samples were collected during 8 hours before and after morning feed and plasma levels of ethanol, lactate, β-hydroxybutyrate (BHBA), insulin, metabolites, GOT and GPT were assayed. The area upper or under the curve (AUC) of blood parameters for 2 and 8 hours were calculated and assesses correlation among the AUCs.

Body weight gain (BWG) was lower ($P < 0.05$) in ewes fed RS-APS. Plasma concentrations of ethanol, lactate and BHBA elevated after feeding of RS-APS ($P < 0.01$, $P < 0.05$ and $P < 0.05$; respectively) and peaked at 2, 1 and 4 hours, respectively. Plasma insulin level tended to increase ($P = 0.06$) after RS-APS feeding but the magnitude of postprandial glucose decline did not differ between treatments. After feeding of RS-APS, plasma F-Cho, HDL-Cho and phospholipid were increased ($P < 0.05$, $P < 0.01$ and $P < 0.1$; respectively) and plasma UN was decreased ($P < 0.05$). The AUCs of plasma ethanol and BHBA in the RS-APS treatment were greater ($P < 0.05$ and $P < 0.01$, respectively) than those of control. The AUC of plasma lactate was tended to grater ($P < 0.1$) and phospholipid AUC was tended to smaller ($P < 0.1$) in RS-APS treatment than those of control. The AUCs of plasma ethanol ($P < 0.01$) and lactate ($P < 0.05$) were positively correlated with that of BHBA. Insulin AUC was positively correlated ($P < 0.01$) with the AUCs of ethanol and BHBA. According to the consistent increase in plasma ethanol and BHBA after feeding of RS-APS, increased lipid profiles for RS-APS group and positive correlation of BHBA with lactate and ethanol, we concluded that APS ethanol consumption induce postprandial transient hyperketonemia through suppression of hepatic gluconeogenesis.

Table 2.1.1: Ingredient compositions of rice straw apple pomace silage[1] (%, on FM basis).

Ingredient	**RS-APS**
Fresh AP[2]	65.0
Soybean meal	2.5
Corn	13.0
Wheat bran	17.0
Rice straw	2.5

[1]A blend of the RS-APS was formulated to have similar TDN content to a commercial concentrates (CP 15.5% and TDN 70%).

[2]Fresh AP within 2 days of production was used.

FM, fresh matter; RS-APS, rice straw apple pomace silage; AP, apple pomace.

Table 2.1.2: Chemical composition of feed ingredients used in the rice straw apple pomace silage fed to ewes.

Item	Fresh AP[1]	Soybean meal	Corn	Wheat bran	Rice straw
Dry matter (%)	18.4	88.3	86.5	88.7	87.8
Nutrient composition (%, DM basis)					
Organic matter	97.8	93.3	98.5	94.2	82.6
Crude protein	5.4	52.2	9.2	17.7	5.4
Crude fiber	16.8	6.3	2.0	10.5	32.3
Ether extract	4.9	1.5	4.4	4.5	2.1
NFE	70.7	33.3	82.9	61.5	42.8
Crude ash	2.2	6.7	1.5	5.8	17.4
ADF	28.3	8.9	3.0	14.2	39.2
NDF	35.9	14.3	10.5	38.8	63.1
Hemicellulose	7.6	5.3	7.5	24.7	23.9
NFC[2]	51.6	25.4	74.3	33.2	12.1

[1]Fresh AP within 2 days of production was used.

[2]NFC = (100 - CP - EE -NDF - Ash).

AP, apple pomace; RS-APS, rice straw apple pomace silage; NFE, nitrogen free extract; ADF, acid detergent fibre; NDF, neutral detergent fibre; NFC, non-fibrous carbohydrate, DM, dry matter.

Table 2.1.3: The pH value, organic acids and others fermented product of apple pomace and rice straw apple pomace silage.

Items	**Fresh AP[1]**	**RS-APS**
pH	4.01	3.6
Organic acids and other fermented products (g kg $^{-1}$ DM)		
Lactic acid	15.3	72.6
Acetic acid	5.0	32.4
Propionic acid	ND	ND
Normal butyric acid	ND	ND
Iso-butyric acid	0.9	ND
Ethanol	1.3	33.4
VBN in TN	0.06	0.12
Moisture	767.0	593.0

[1]Fresh apple pomace within 2 days of production was used.

AP, apple pomace; RS-APS, rice straw apple pomace silage; DM, dry matter; ND, not detectable; VBN, volatile basic nitrogen; TN, total nitrogen.

Table 2.1.4: Chemical composition of experimental feeds.

Items	Alfalfa hay cube	Concentrate	RS-APS
Dry matter (%)	91.5 ± 1.9	88.2 ± 0.1	40.0 ± 0.2
Composition (%, DM basis)			
Organic matter	87.7 ± 0.6	93.2 ± 0.2	95.7 ± 0.2
Crude protein	17.7 ± 0.4	17.1 ± 0.3	14.4 ± 0.4
Ether extract	2.0 ± 0.1	3.2 ± 0.02	5.5 ± 0.02
Crude ash	12.3 ± 0.4	6.8 ± 0.2	4.4 ± 0.1
ADF	31.9 ± 0.2	3.6 ± 0.1	13.2 ± 0.5
NDF	44.6 ± 0.8	20.7 ± 0.5	34.7 ± 1.4
Hemicellulose	12.7 ± 0.4	17.1 ± 0.04	21.6 ± 0.8
NFC[1]	31.6 ± 0.1	56.7 ± 0.3	52.1 ± 1.8

[1]NFC = (100 - CP - EE -NDF - Ash).

RS-APS, rice straw apple pomace silage; DM, dry matter; ADF, acid detergent fiber; NDF, neutral detergent fiber; NFC, non-fibrous carbohydrate.

Table 2.1.5: Body weights and performance traits in the ewes [1].

Items	Control	RS-APS	SE	P-value
Initial BW, kg	38.9	39.1	1.6	0.83
Final BW, kg	44.3	43.9	1.6	0.60
Average BWG, g/d	190	174	2.3	0.04
Average TDNI, g/d	812	805	6.8	0.54
FCR (TDNI/BWG)	4.3	4.7	0.1	0.06
Ethanol intake[2], g/d	ND	22.8	0.2	<0.001

[1]Values are lsmeans of four ewes per treatment.

[2]Ethanol intake was calculated from RS-APS intake and ethanol contents of the RS-APS.

RS-APS, rice straw apple pomace silage; SE, standard error of the mean; ND, not detectable; BW, body weight; BWG, body weight gain; TDNI, total digestible nutrient intake; FCR, feed conversion ratio.

Table 2.1.6: The response of area upper or under curve (AUC) of plasma parameters for 8 hours sampling windows of ewes fed different experimental diets[1].

Trait	Control	RS-APS	SE	P-value
Ethanol (mmol.min.L^{-1})	-47	688	101.2	0.036
Lactate (mmol.min.L^{-1})	-221	165	83.7	0.082
BHBA (mmol.min.L^{-1})	141	474	30.3	0.016
Insulin (μg.min.L^{-1})	19	50	60.9	0.754
Glucose (mg.min.dL^{-1})	-2960	-7090	1892.4	0.263
T-Cho (mg.min.dL^{-1})	-2329	-1820	278.6	0.326
F-Cho (mg.min.dL^{-1})	-399	-489	67.6	0.448
HDL-Cho (mg.min.dL^{-1})	-295	-1410	1045.2	0.529
Triglyceride (mg.min.dL^{-1})	-3047	-425	651.7	0.105
Phospholipid (mg.min.dL^{-1})	-3743	-1952	421.4	0.095
Plasma UN (mg.min.dL^{-1})	-881	117	374.5	0.20
NEFA (μEq.min.L^{-1})	-89583	-65836	21601.0	0.518
GOT (IU.min.L^{-1})	1470	-10	835.2	0.337
GPT (IU.min.L^{-1})	-78	58	131.2	0.54

[1]Values are lsmeans of four ewes per treatment.

RS-APS, rice straw apple pomace silage; SE, standard error of the mean; AUC, area upper or under the curve; BHBA, β-hydroxybutyrate; T-Cho, total cholesterol; F-Cho, free cholesterol; HDL-Cho, high density lipoprotein cholesterol; UN, urea nitrogen; NEFA, non-esterified fatty acids; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase.

Table 2.1.7: Correlation coefficients among the area upper or under the curve (AUC) for 0-2 hours of plasma ethanol, lactate, β-hydroxybutyrate, insulin, plasma metabolites, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase.

Items	Ethanol	Lactate	BHBA	Insulin	Glucose
Lactate	0.68				
BHBA	0.87**	0.82*			
Insulin	0.94**	0.52	0.86**		
Glucose	-0.60	-0.48	-0.39	-0.39	
T-Cho	0.34	-0.03	0.45	0.59	0.31
F-Cho	-0.08	0.12	0.29	0.12	0.47
HDL-Cho	0.23	0.35	0.43	0.25	0.40
Triglyceride	0.37	0.40	0.36	0.35	0.05
Phospholipid	0.23	0.09	0.44	0.48	0.18
Plasma UN	0.14	0.21	0.04	0.08	0.09
NEFA	0.18	0.09	0.06	0.10	-0.24
GOT	0.25	0.06	0.41	0.33	0.13
GPT	0.55	0.53	0.74*	0.63	-0.04

[1]Correlations were calculated on data sets of 8 observations.

[2]The asterisk(s) indicate statistical significance (*P < 0.05 and **P < 0.01).

AUC, area upper or under the curve; BHBA, β-hydroxybutyrate; T-Cho, total cholesterol; F-Cho, free cholesterol; HDL-Cho, high density lipoprotein cholesterol; UN, urea nitrogen; NEFA, non-esterified fatty acids; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase.

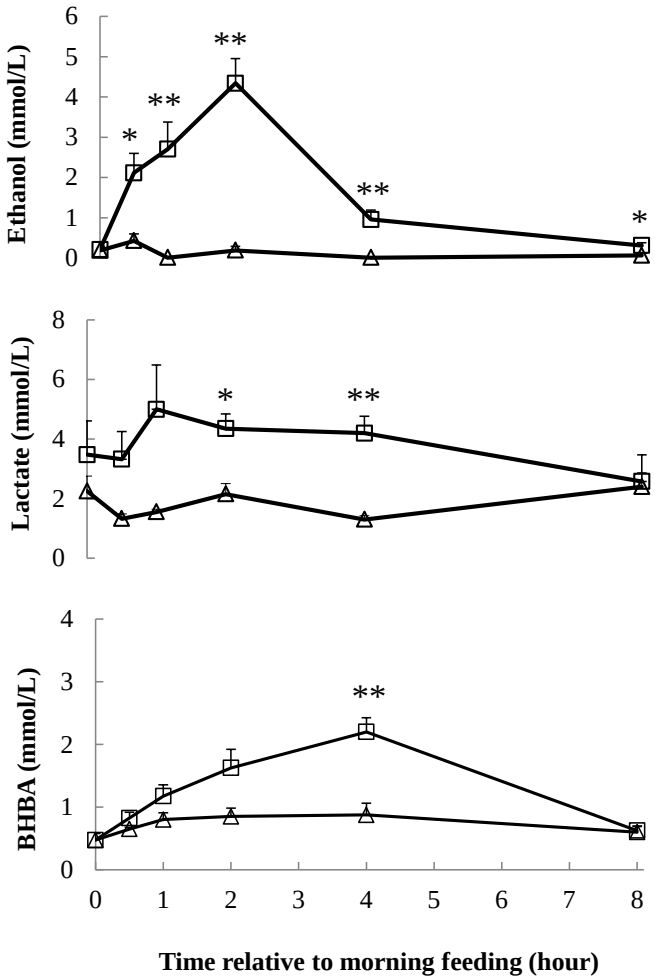

Figure 2.1.1: Time course changes of plasma Ethanol, Lactate and β-hydroxybutyrate (BHBA) of ewes fed on control (Δ) or RS-APS (□) treatment. The ethanol intake in RS-APS group was calculated to be 22.8 g/d/head. Each data point is mean of 4 observations ± standard error of the means. The asterisk(s) indicate the significant difference(s) between treatments at each time point (*P < 0.05 and **P < 0.01).

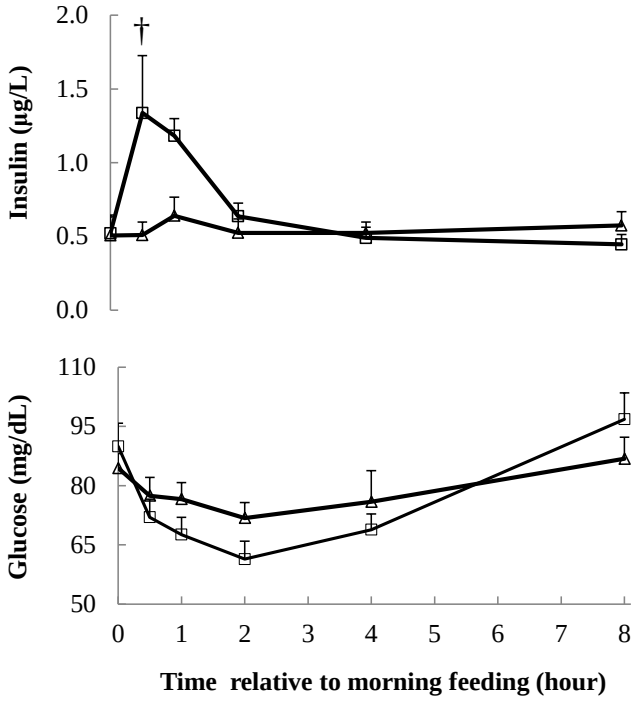

Figure 2.1.2: Time course changes of plasma Insulin and Glucose of ewes fed on control (Δ) or RS-APS (□) tretament. The ethanol intake in RS-APS group was calculated to be 22.8 g/d/head. Each data point is mean of 4 observations ± standard error of the means. The dagger indicate a tendency toward significant difference between treatments at the time point (†$P < 0.1$).

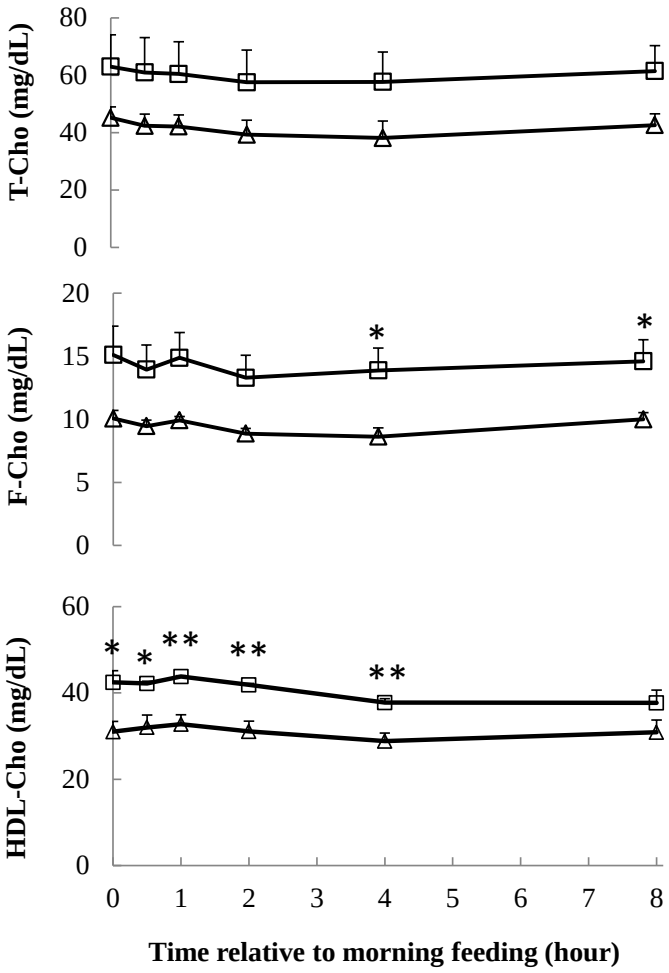

Figure 2.1.3: Time course changes of plasma Total-Cholesterol (T-Cho), Free-Cholesterol (F-Cho) and High density lipoprotein Cholesterol (HDL-Cho) of ewes fed on control (Δ) or RS-APS (□) treatment. The ethanol intake in RS-APS group was calculated to be 22.8 g/d/head. Each data point is mean of 4 observations ± standard error of the means. The asterisk(s) indicate the significant difference(s) between treatments at each time point (*P < 0.05 and **P < 0.01).

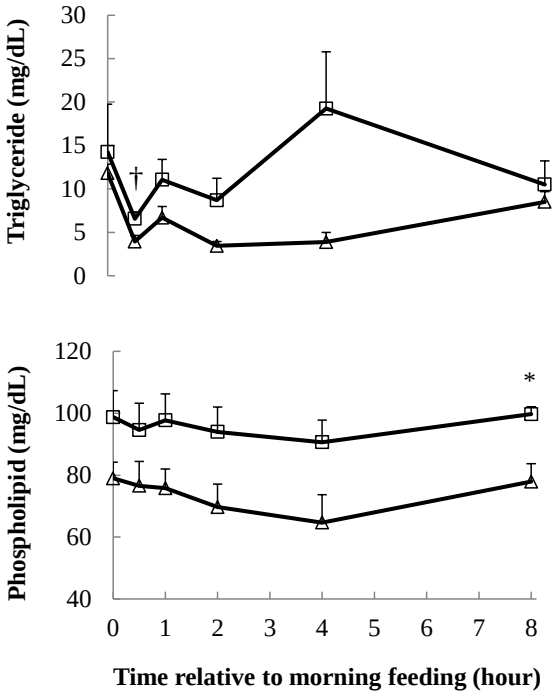

Figure 2.1.4: Time course changes of plasma Triglyceride and Phospholipid of ewes fed on control (Δ) or RS-APS (□) traetment. The ethanol intake in RS-APS group was calculated to be 22.8 g/d/head. Each data point is mean of 4 observations ± standard error of the means. The dagger or asterisk(s) indicate a tendency toward significant difference or the significant difference (s) between treatments at each time point (†P < 0.1 and *P < 0.05).

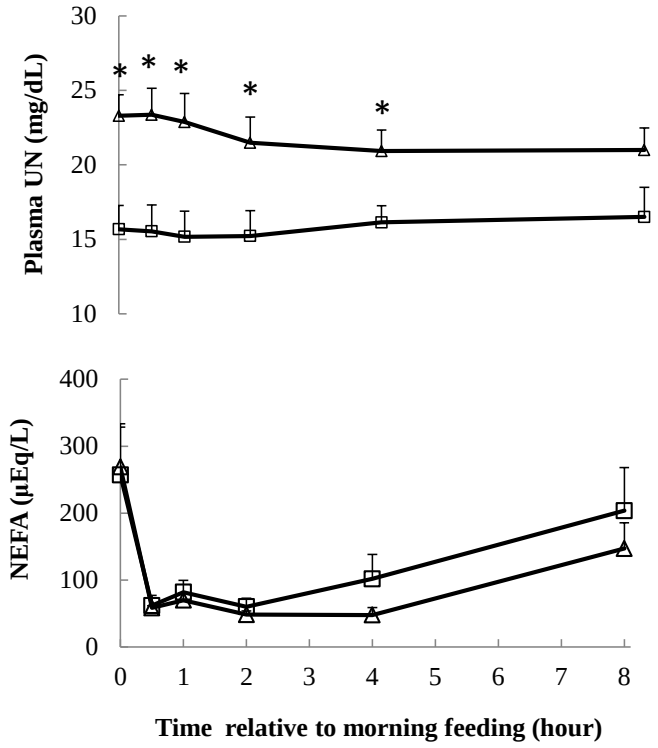

Figure 2.1.5: Time course changes of plasma urea nitrogen (UN) and non-esterified fatty acids (NEFA) of ewes fed on control (Δ) or RS-APS (□) treatment. The ethanol intake in RS-APS group was calculated to be 22.8 g/d/head. Each data point is mean of 4 observations ± standard error of the means. The asterisk(s) indicate the significant difference(s) between treatments at each time point (*P < 0.05).

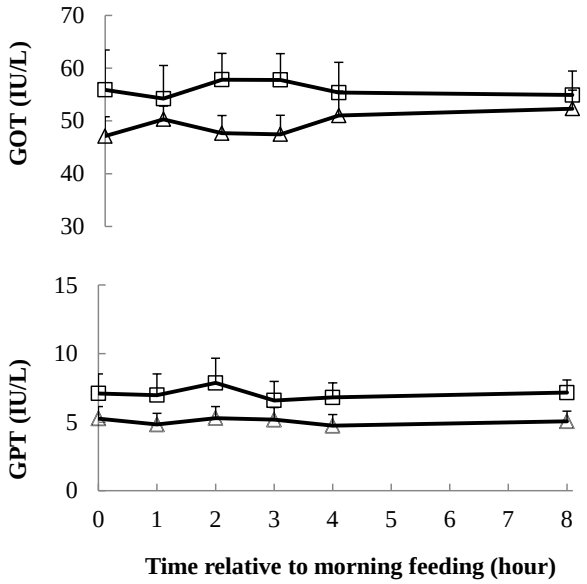

Figure 2.1.6: Time course changes for the enzyme activity of plasma glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) of ewes fed on control (Δ) or RS-APS (□) treatment. The ethanol intake in RS-APS group was calculated to be 22.8 g/d/head. Each data point is mean of 4 observations ± standard error of the means.

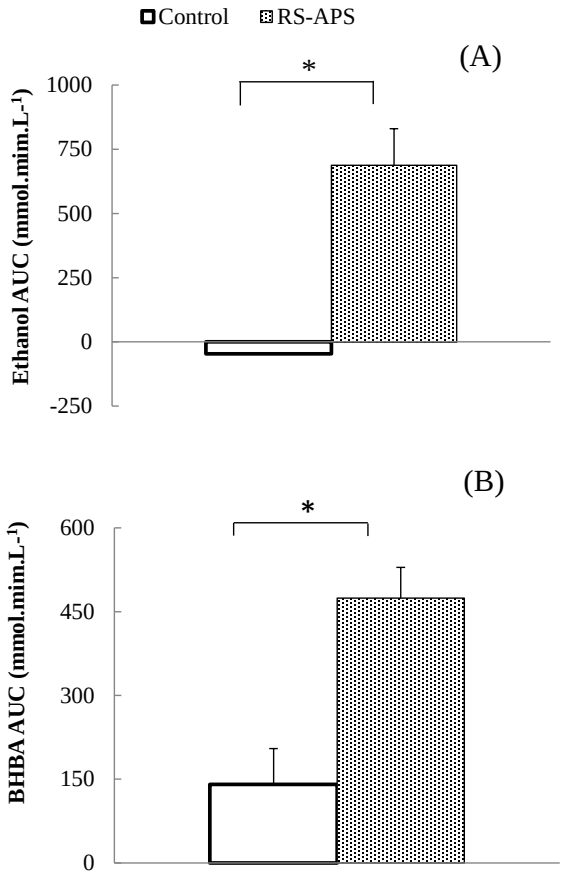

Figure 2.1.7: The area upper or under the curve (AUC) of plasma ethanol (A) and β-hydroxybutyrate (BHBA) (B) of ewes fed on control treatment (open bar**)** or RS-APS treatment (dotted bar) for 8 hours sampling windows. Each data point is the mean of 4 observations ± standard error of the means. The asterisk(s) indicate a significant difference between treatments (*P < 0.05).

CHAPTER 2.2

Effect of feeding low and high levels ethanol containing apple pomace silages on response of blood parameters in Suffolk ewes

INTRODUCTION

Ethanol is often produced in significant amounts during conservation by fermentation of forages or by-product (Jean-Blain et al., 1991). Moreover, ensiling apple pomace (AP) results in the conversion of most of the water-soluble carbohydrates into fermentation end-products (McDonald et al., 1991). Ethanol is the main fermentation end-product in apple pomace silages (APS) and which contained a considerable amount of ethanol reported by Alibes et al., 1984 (50.5 and 173.3 g kg^{-1} DM for fresh AP and APS were, respectively). In ruminants, dietary ethanol is partially metabolized by rumen microbes (Durix et al., 1991; Jean-Blain et al., 1992; Raun and Kristensen, 2011**)** and is also immediately absorbed across rumen epithelium (Veresegyházy et al., 2003). Excess alcohol intake may result in increased alcohol load into the liver (Jean-Blain et al., 1992) and followed by hours of elevated peripheral concentrations of alcohol (Kristensen et al., 2007). Dietary ethanol is associated with ethanol-induce hypoglycemia in human (Brand-Miller et al., 2007) and the hypoglycemic effect of ethanol with APS ingestion in sheep was first described by Kondo et al. (2011). Subsequently, it was confirmed that APS ethanol induces postprandial transient hyperketonemia through suppression of hepatic gluconeogenesis by the previous study (Chapter 2.1). But the information on the impact of over doses APSs ethanol on blood biochemistry is limited and need to clarify. Under such conditions of suppressed gluconeogenesis, gluconeogenic precursors including lactate in the circulation would be elevated. Concomitant problems of hypoglycemia and hyperlactatemia might lead to hyperketonemia and the end up in ketosis. Therefore, the current study was undertaken to investigate the effect of feeding effects of different

types alcoholic fermented APSs that are rich in ethanol on blood parameters in Suffolk ewes.

MATERIALS AND METHODS

1. Animals, experimental design and management

All procedures involving animals in this study were approved by the Institutional Animal Care and Use Committee at Hirosaki University (A08023).

Four Suffolk shorn ewes, aged 14 months on average, weighing 60.3 ± 3.8 kg, were used in this experiment to investigate the comparison of low and high ethanol APS (44.1g *vs.* 66.9 g of ethanol/kg DM) feeding effect on response of blood parameters in Suffolk ewes. The experiment was performed using crossover design over two 35-day periods including of initial 7 days for dietary adaptation. In this experiment, ewes were allocated to two dietary treatments; alfalfa hay cube and either low ethanol containing APS (L-APS) or high ethanol containing APS (H-APS) at a ratio of 30:70 on TDN basis. During the first period two ewes were fed on L-APS diet and then fed on H-APS diet during the second period. The other two ewes received the treatment diets in reverse order. Daily allowance was offered as TDN requirement for maintenance and daily 100g gain (Ministry of Agriculture, Forestry and Fisheries, 1996) at 10:00 and 18:00 in 2 equal meals. The required amount was adjusted weekly based on their BW. Ewes were housed in individual pens with sawdust bedding in an indoor animal room and had free access to a trace mineralized salt block and water throughout the experiment.

2. Apple pomace silage preparation

Fresh apple pomace (AP) was obtained from an apple juice factory of the farm village industry federation of Aomori prefectural agricultural cooperatives at Hirosaki, Japan. Low ethanol containing APS (L-APS) was prepared with fresh AP, soybean meal, wheat bran and beet pulp which was formulated to balance nutrient contents similar to the commercial concentrates (Table 2.2.1). The high ethanol containing APS

(H-APS) was also prepared with the intension of having the same overall nutrient composition except of fermented AP for the different ethanol content. Because remaining sugars in fresh AP is fermented rapidly and efficiently turned into ethanol under solid-state fermentation condition (Hang et al. 1981), fresh AP was stored alone in an anaerobic condition for 2 months to secure higher ethanol contents before ensiling of H-APS. For ensiling of L-APS, fresh AP within 2 days of production was used. Apple pomace and all other ingredients were mixed thoroughly by a mechanical mixer and stuffed into a plastic container, pressed sufficient to fill properly, topped with air-tight cover and ensiled for at least 8 months before use in the feeding experiment.

3. Feed samples and analyses

Apple pomace silage extraction procedure and determination procedure of pH, VBN, organic acid and ethanol were same as described in the previous part (Chapter 2.1). All feed samples were dried in a forced-air oven at 60 °C for 48 hours, ground through a 1mm screen using a Willey mill and kept in a plastic airtight container for chemical analyses. Dry matter (DM), crude protein (CP), crude fiber (CF), ether extract (EE), ash, neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by following the methods as described in the previous part (Chapter 2.1).

Chemical compositions of feed ingredients used in the APSs are presented in Table 2.2.2.The pH value, organic acids and other fermented products in AP and APSs are presented in Table 2.2.3. Chemical compositions of experimental feeds are presented in Table 2.2.4. Fermented AP contained the largest amount of ethanol (Table 2.2.3) and thus ethanol contents of L-APS and H-APS were successfully differentiated.

4. Blood samples collection and analytical methods

On the last day of each 35 day period, series of blood samples were obtained by jugular venipuncture at pre-feeding (0) and at 0.5, 1, 2, 4 and 8 hours after the morning feed. The blood was collected into two 7 mL evacuated tubes containing sodium heparin as an anticoagulant, chilled on ice and centrifuged (3000 × g for 15 min at 4 °C) to harvest plasma sample. Plasma was aliquotted and stored at -30 °C until

analyzed for ethanol, lactate, β-hydroxybutyrate (BHBA), insulin and blood metabolites [glucose, non-esterified fatty acids (NEFA), plasma urea nitrogen (UN), total cholesterol (T-Cho), free cholesterol (F-Cho), high density lipoprotein cholesterol (HDL-Cho), phospholipid and triglyceride]. Another aliquot was stored at -80 °C until analysed for the ethanol-induced liver injury biochemical marker of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT).

All plasma samples were assayed by the following procedures as described in the previous part (Chapter 2.1). The area upper or under the curve (AUC) for 2 and 8 hours after feeding of plasma parameters was calculated as an indicative of the responses to feeding in each parameters.

5. Calculation

Calculation procedure of hemicellulose, NFC, BWG, TDN intake and FCR were same as described in the chapter 2.1. Ethanol intake was calculated from APS intake and ethanol content of the APS.

6. Statistical analysis

Performance traits data were analyzed using the general linear model (GLM) procedure of SAS (2003). Sheep, period and treatment were included in the model. Treatment means were separated using a PDIFF option, protected by overall F-test. Difference was considered statistically significant at $P < 0.05$ and considered a tendency toward significance at $0.05 \leq P < 0.1$. Serial data for blood parameters were analyzed with repeated measures ANOVA using GLM procedure of SAS. If the treatment effect or treatment × time interaction was significant, treatment means within each sampling time were separated by least significant difference procedure. Relationship among the AUC responses for plasma measurements was analyzed by Pearson's correlation coefficient.

RESULTS

1. Performance traits

No health problems were observed in any of ewes throughout this experiment also. Lsmeans for body weights and performance traits of the ewes are presented in Table 2.2.5. No difference was observed in BWs, average BWG, TDN intake and FCR, although ethanol intake was significantly greater ($P < 0.01$) in the ewes fed on H-APS than L-APS treatment.

2. Blood parameters

Time course changes of plasma ethanol, lactate and BHBA concentrations are presented in Figure 2.2.1. After feeding of both L-APS and H-APS, plasma concentrations of ethanol, lactate and BHBA were elevated ($P < 0.01$) and peaked at 2, 4 and 4 hours, respectively, however no significant treatment effect were detected.

Time course changes of plasma insulin and glucose are illustrated in Figure 2.2.2. Plasma insulin concentration showed non-significant temporal increase immediately after feeding of APSs, but the level was not affected by ethanol content of APS. Plasma glucose concentration of both groups were declined ($P < 0.05$) over the first 2 to 4 hours and returned to pre-feeding levels at 8 hours after the morning feed, however no significant treatment effect was detected.

Time course changes of plasma T-Cho, F-Cho and HDL-Cho are illustrated in Figure 2.2.3. After feeding of both APSs, plasma T-Cho concentrations were increased ($P < 0.01$) and peaked at 0.5 hour, however no significant treatment effect was detected. After feeding of L-APS and H-APS, plasma concentrations of HDL-Cho were declined ($P < 0.01$) over the 2 and 4 hours, respectively, however, no significant difference was detected. Plasma F-Cho concentration showed non-significant temporal increase immediately after feeding of H-APS, but no significant difference was detected.

Time course changes of plasma triglyceride and phospholipid are illustrated in Figure 2.2.4. After feeding of both L-APS and H-APS, plasma triglyceride and phospholipid concentrations were not differ significantly between the treatments.

Time course changes of plasma UN and NEFA are illustrated in Figure 2.2.5. After feeding, plasma UN and NEFA concentrations were not differ significantly between the L-APS and H-APS treatments.

Time course changes for the enzyme activity of plasma GOT and GPT are illustrated in Figure 2.2.6. After feeding of the both APSs, no significant treatment effect was detected for the plasma enzyme activity of GOT and GPT.

3. Response of area upper or under curve (AUC)

The AUC of plasma parameters for 8 hours sampling windows are represent in Table 2.2.6. The AUC of plasma HDL-Cho was tended to greater ($P < 0.1$) for H-APS treatment than that of L-APS. The AUC of plasma phospholipid was greater ($P < 0.05$) for L-APS treatment than that of H-APS (Figure 2.2.7). No significant differences were observed between the L-APS and H-APS treatments for the AUCs of plasma ethanol, lactate, BHBA, insulin, glucose, T-Cho, F-Cho, TG, NEFA, BUN, GOT and GPT.

4. Correlations among AUC responses of plasma measurements

The correlation coefficients among AUCs of plasma ethanol, lactate, BHBA, insulin, plasma metabolites, GOT and GPT during 2 hours after feeding are presented in Table 2.2.7.

The AUC of lactate was positively ($P < 0.01$) correlated with those of BHBA and triglyceride. The BHBA AUC was also positively ($P < 0.05$) correlated with that of triglyceride. The glucose AUC was negatively ($P < 0.05$) correlated with AUCs of lactate and BHBA. The AUCs of ethanol and insulin were not correlated with any of the AUCs for plasma measurement.

DISCUSSION

In the present study, largest ethanol content was observed in fermented AP in comparison with APSs that blended with other ingredients to improve nutritional balance of AP after 2 month storage period under similar anaerobic condition. The

fermented AP contained a comparable amount of ethanol (243 g/kg DM) in a single ingredient APS (173 g/kg DM) reported by Alibes et al. (1984). By using fermented AP for preparation of H-APS, we succeeded to increase its ethanol content by 52% compared to that of L-APS. The L-APS contained some 2 - 3 times greater ethanol content compared to corn, grass and TMR silages (McDonald et al., 1991; Raun and Kristensen, 2010; Lawrence et al., 2011; Cummins et al. 2009).

A slight non-significant reduction in average BWG was observed in the H-APS compared to L-APS treatment in this experiment. We have formulated daily ration to meet the daily TDN requirement for maintenance and daily 100g gain of growing ewes (Ministry of Agriculture, Forestry and Fisheries, 1996; Japan) where APSs provided a large amount (70%) of TDN requirement, probably high moisture content of H-APS influences the slightly lower BWG. But any of performance traits was not affected by a difference in ethanol intake between L-APS and H-APS treatments in this experiment. Therefore, ingested dose of ethanol (50 g/d) from APSs in this study would not cause any harmful influence on performance of growing ewes.

The plasma ethanol, lactate and BHBA concentrations were elevated after feeding of APSs and returned to pre-feeding levels until 8 hours of morning feed. In the previous experiment (chapter 2.1) was also found the simultaneous postprandial increases in plasma ethanol, lactate and BHBA levels by feeding alcoholic fermented APSs. Kristensen et al. (2007) also observed the similar increases trend in plasma concentrations of ethanol, lactate and BHBA after feeding of corn silages (14.2 g/kg DM ethanol). As Alibes et al. (1984) cautioned against overfeeding of alcoholic fermented APS, ethanol content of H-APS used in the present study was more than at least 4.5 times as much as corn silage (McDonald et al., 1991; Kristensen et al., 2007; Raun and Kristensen, 2010). Moreover, in this study, ewes received a higher amount of APSs about 2500 to 3000 g/d (fresh basis). Nonetheless all of the blood parameters determined in this study returned to pre-feeding levels until 8 hours after morning feed and none of clinical signs of health problem was observed. According to the results of kinetic study of infused ethanol in sheep, Jean-Blain et al. (1992) assumed that daily

ethanol intake ranging from 0.2 to 1 g/kg BW can be metabolized by rumen microflora and enzymatic system of the host and plasma ethanol level remains below 0.25 g/L. These estimates are correspond very well to the fact that ewes consumed 0.6 ~ 0.9 g/kg BW of ethanol and the highest plasma ethanol level was 0.17 g/L observed in the present study. Increased dose of ethanol in the H-APS treatment in this experiment did not produce any augmented response in blood parameters. This is probably because of ethanol intake in the H-APS treatment was within the aforementioned metabolizable range.

It is established that ethanol ingestion resultant of hypoglycemia (Krebs et al., 1969; Brand-Miller et al., 2007) which decrease in the cytosolic free NAD^+ to NADH ratio and this change in the redox state decreases the concentration of pyruvate and other gluconeogenic intermediates (Krebs et al., 1969) and thus cause postprandial hypoglycemia in sheep fed on APS diets (Kondo et al., 2010; 2011). The negative effects of NADH: NAD^+ on hepatic gluconeogenesis, fatty acid oxidation is reduced. Accompanying with the combination of higher dietary fat and ethanol has a synergistic effect in tended to increase plasma lipid concentration because ethanol suppresses the clearance of intestinally derived chylomicrons. Holtenius and Holtenius (1996) reported that primary factor for the ketotic state is the high demand for glucose and insufficient gluconeogenesis, which is compensated by increased ketogenesis, there are very few prerequisites for lipid synthesis in the liver cell and small risks for fat accumulation for ruminants. Accumulation of fat in the liver can be alleviated by secreting lipids into the blood steam. The grater AUC of plasma phospholipid in L-APS treatment and the consistent positive correlations between plasma triglyceride AUC and the AUCs of plasma lactate and BHBA levels in this experiment are strongly supporting this notion. Although we failed to demonstrate significant treatment effect in plasma ethanol, lactate and BHBA concentrations in the APSs, however, negative correlations between plasma glucose AUC and the AUCs of plasma lactate and BHBA levels in this experiment may also reflected ethanol-suppressed gluconeogenesis and resultant hypoglycemia in APS consumed animals. Therefore once ingested ethanol

suppressed gluconeogenesis, lactate would become redundant in gluconeogenic pathway and be accumulated and BHBA would also be generated from fatty acids in well-fed ruminant animals. Consistent positive correlations between the AUCs of plasma lactate and BHBA concentrations in this experiment are strongly supporting this notion again. Although all of the responses observed in blood parameters are transient in this study and no health problems were detected, need for hepatic tissue observation to assess safety and productivity of the use of APS is clearly warranted.

In conclusion, according to the consistent positive correlation of postprandial BHBA with lactate responses and negative correlation of changes in glucose with those in lactate and BHBA after APSs feeding, we concluded that APS ethanol consumption induce postprandial hypoglycemia which influence transient hyperketonemia through suppression of hepatic gluconeogenesis.

SUMMARY

The present experiment was to investigate the comparison of low and high ethanol APS (44.1g *vs.* 66.9 g of ethanol/kg DM) feeding effect of APSs on response of blood parameters in Suffolk ewes. Four ewes were fed alfalfa hay cube and either L-APS or H-APS at 30:70 of TDN requirement for maintenance and daily 100g gain in a 2 × 2 crossover design. On the last day of each 35 days period, blood samples were collected before and after the morning feed for 8 hours. All plasma samples were assayed for ethanol, lactate, β-hydroxybutyrate (BHBA), insulin, glucose, total cholesterol (T-cho), free cholesterol (F-Cho), high density lipoprotein cholesterol (HDL-Cho), triglyceride, phospholipid, plasma urea nitrogen (UN), non-esterified fatty acids (NEFA), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT). The area upper or under the curve (AUC) for 2 and 8 hours and correlation among the AUCs responses were calculated.

The changes in the blood parameters showed similar trends after feeding of two APSs but no significant treatment effect was detected. The AUC of plasma phospholipid was greater ($P < 0.05$) for L-APS treatment than that of H-APS. The AUC

of lactate was positively ($P < 0.01$) correlated with those of BHBA and triglyceride. The BHBA AUC was also positively ($P < 0.05$) correlated with that of triglyceride. The glucose AUC was negatively ($P < 0.05$) correlated with AUCs of lactate and BHBA.

According to the consistent positive correlation of postprandial BHBA with lactate responses and negative correlation of changes in glucose with those in lactate and BHBA after APSs feeding, we concluded that APS ethanol consumption induce postprandial hypoglycemia which influence transient hyperketonemia through suppression of hepatic gluconeogenesis.

Table 2.2.1: Ingredient compositions of apple pomace silages[1] (%, fresh basis).

Ingredient	L-APS	H-APS
Fresh AP[2]	70.0	-
Fermented AP[3]	-	70.0
Soyabean meal	6.0	6.0
Wheat bran	12.0	12.0
Beet pulp	12.0	12.0

[1]A blend of the L-APS/H-APS was formulated to have similar TDN content to a commercial concentrates (CP 15.5% and TDN 70%).

[2]Fresh apple pomace within 2 days of production was used.

[3]Fermented apple pomace stored anaerobically for 2 months after production was used.

AP, apple pomace; L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage.

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Table 2.2.2: Chemical composition of feed ingredients used in the apple pomace silages fed to ewes.

Item	Fresh AP[1]	Fermented AP[2]	Beet pulp	Soybean meal	Wheat bran
Dry matter (%)	21.4 ± 0.1	14.4 ± 0.2	92.0 ± 2.5	92.1 ± 3.2	91.8 ± 4.8
Nutrient composition (%, DM basis)					
Organic matter	97.7 ± 0.1	96.3 ± 0.2	93.9 ± 0.2	92.9 ± 0.2	94.2 ± 0.2
Crude protein	4.5 ± 0.2	7.5 ± 0.3	6.9 ± 0.4	47.9 ± 0.4	17.4 ± 0.4
Crude fiber	13.7 ± 0.4	21.1 ± 0.2	18.5 ± 0.3	5.6 ± 0.3	9.2 ± 0.3
Ether extract	3.2 ± 0.0	5.9 ± 0.0	0.7 ± 0.0	1.5 ± 0.0	4.7 ± 0.0
NFE	76.3 ± 0.7	61.8 ± 0.3	67.7 ± 0.9	37.8 ± 0.9	62.8 ± 0.9
Crude ash	2.3 ± 0.0	3.7 ± 0.2	6.1 ± 0.1	7.1 ± 0.1	5.8 ± 0.1
ADF	20.3 ± 0.1	31.4 ± 0.6	25.3 ± 0.3	8.3 ± 0.3	12.6 ± 0.3
NDF	27.9 ± 1.2	42.5 ± 0.2	38.2 ± 0.2	11.8 ± 2.6	41.9 ± 1.7
Hemicellulose	7.5 ± 1.3	11.1 ± 0.4	12.8 ± 0.5	3.4 ± 2.8	29.3 ± 2.0
NFC[3]	62.1 ± 1.4	40.4 ± 0.4	48.1 ± 0.4	31.7 ± 1.5	30.2 ± 1.7

[1]Fresh apple pomace within 2 days of production was used.

[2]Fermented AP was stored fresh AP for 2 months in an anaerobic condition.

[3]NFC = (100 - CP - EE - NDF - Ash).

AP, apple pomace; L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; DM, dry matter; NFE, nitrogen free extract; ADF, acid detergent fibre; NDF, neutral detergent fibre; NFC, non-fibrous carbohydrate.

Table 2.2.3: The pH value, organic acids and others fermented product of apple pomace and apple pomace silage.

Item	Fresh AP[1]	Fermented AP[2]	L-APS	H-APS
pH	4.01	4.08	3.86	3.75
Organic acids and other fermented products (g kg $^{-1}$ DM)				
Lactic acid	15.3	72.9	30.7	37.1
Acetic acid	5.0	40.6	13.4	17.9
Iso-butyric acid	0.9	ND	ND	ND
Butyric acid	ND	ND	ND	ND
Propionic acid	ND	ND	ND	ND
Ethanol	1.3	242.5	44.1	66.9
VBN in TN	0.06	0.13	0.78	1.23
Moisture	767.0	856.9	630.1	661.7

[1]Fresh apple pomace within 2 days of production was used.

[2]Fermented AP was stored fresh AP for 2 months in an anaerobic condition.

AP, apple pomace; L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; DM, dry matter; ND, not detectable; VBN, volatile basic nitrogen; TN, total nitrogen.

Table 2.2.4: Chemical composition of experimental feeds.

Items	**Alfalfa hay cube**	**L-APS**	**H-APS**
Dry matter (%)	91.9 ± 0.4	37.3 ± 0.2	33.3 ± 2.1
***Nutrients composition* (*%, DM basis*)**			
Organic matter	87.5 ± 0.5	94.5 ± 0.0	94.1 ± 0.1
Crude protein	17.7 ± 0.2	17.2 ± 0.5	18.6 ± 0.6
Ether extract	2.0 ± 0.1	3.2 ± 0.1	3.8 ± 0.1
Crude ash	12.5 ± 0.2	5.5 ± 0.1	5.9 ± 0.2
ADF	30.7 ± 1.4	19.9 ± 0.5	19.5 ± 0.5
NDF	43.4 ± 1.3	35.8 ± 1.2	37.6 ± 0.3
Hemicellulose	12.7 ± 1.3	15.9 ± 1.7	18.1 ± 0.1
NFC[1]	31.4 ± 0.4	48.4 ± 2.1	48.2 ± 0.6

[1]NFC = (100 - CP - EE -NDF - Ash).

L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; DM, dry matter; ADF, acid detergent fibre; NDF, neutral detergent fibre; NFC, non-fibrous carbohydrate, %, percent.

Table 2.2.5: Body weights and performance traits in the ewes [1].

Items	L-APS	H-APS	SE	P-value
Initial BW, kg	57.8	57.9	0.3	0.77
Final BW, kg	62.9	62.6	0.3	0.55
Average BWG, g/d	185	170	6.8	0.27
Average TDNI, g/d	1093	1077	13.9	0.50
FCR (TDNI/BWG)	6.1	6.7	0.4	0.44
Ethanol intake[2], g/d	37.1	50.1	0.7	<0.01

[1] Values are lsmeans of four ewes per treatment.

[2] Ethanol intakes were calculated from APS intake and ethanol contents of the APSs. L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; SE, standard error of the mean; BW, body weight; BWG, body weight gain; TDNI, total digestible nutrient intake; FCR, feed conversion ratio; g, gram; kg, kilogram; d, day.

Table 2.2.6: The response of area upper or under curve of plasma parameters for 8 hours sampling windows of ewes fed different experimental diets[1].

Trait	L-APS	H-APS	SE	P-value
Ethanol (mmol.min.L^{-1})	515	831	159.8	0.296
Lactate (mmol.min.L^{-1})	942	1111	78.2	0.265
BHBA (mmol.min.L^{-1})	792	765	12.5	0.265
Insulin (μg.min.L^{-1})	12	41	151	0.904
Glucose (mg.min.dL^{-1})	-7674	-3790	1669.1	0.242
T-Cho (mg.min.dL^{-1})	-1429	-1221	826.6	0.875
F-Cho (mg.min.dL^{-1})	-227	-1021	696.1	0.505
HDL-Cho (mg.min.dL^{-1})	-3106	-4726	384.4	0.096
Triglyceride (mg.min.dL^{-1})	1422	1542	247.9	0.764
Phospholipid (mg.min.dL^{-1})	-4869	-3142	181.2	0.021
Plasma UN (mg.min.dL^{-1})	-269	-775	349.3	0.414
NEFA (μEq.min.L^{-1})	-40619	-64359	12901	0.323
GOT (IU.min.L^{-1})	-1748	1188	1540.5	0.310
GPT (IU.min.L^{-1})	-90.4	27.8	81.1	0.411

[1] Values are lsmeans of four ewes per treatment.

L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; SE, standard error of the mean; AUC, area upper or under the curve; BHBA, β-hydroxybutyrate; T-Cho, total cholesterol; F-Cho, free cholesterol; HDL-Cho, high density lipoprotein cholesterol; NEFA, non-esterified fatty acids; UN, urea nitrogen; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase.

Table 2.2.7: Correlation coefficients among the area upper or under the curve for 0-2 hours of plasma ethanol, lactate, β-hydroxybutyrate, Insulin, plasma metabolites, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase[1,2].

Items	Ethanol	Lactate	BHBA	Insulin	Glucose
Lactate	0.64				
BHBA	0.69	0.95**			
Insulin	0.29	0.61	0.57		
Glucose	-0.66	-0.75*	-0.72*	-0.30	
T-Cho	-0.23	-0.17	-0.28	-0.14	0.30
F-Cho	-0.06	-0.11	0.15	0.17	0.05
HDL-Cho	0.10	0.00	-0.13	-0.54	-0.35
Triglyceride	0.65	0.84**	0.81*	0.49	-0.64
Phospholipid	0.27	0.03	-0.07	-0.15	-0.08
Plasma UN	-0.54	-0.61	-0.51	-0.26	0.59
NEFA	0.11	-0.08	-0.01	-0.52	-0.03
GOT	0.38	0.20	0.10	0.02	-0.27
GPT	0.64	0.27	0.27	-0.17	-0.64

[1] Correlations were calculated on data sets of 8 observations.

[2]The asterisk(s) indicate statistical significance (*P < 0.05 and **P < 0.01).

AUC, area upper or under the curve; BHBA, β-hydroxybutyrate; T-Cho, total cholesterol; F-Cho, free cholesterol; HDL-Cho, high density lipoprotein cholesterol; NEFA, non-esterified fatty acids; UN, urea nitrogen; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase.

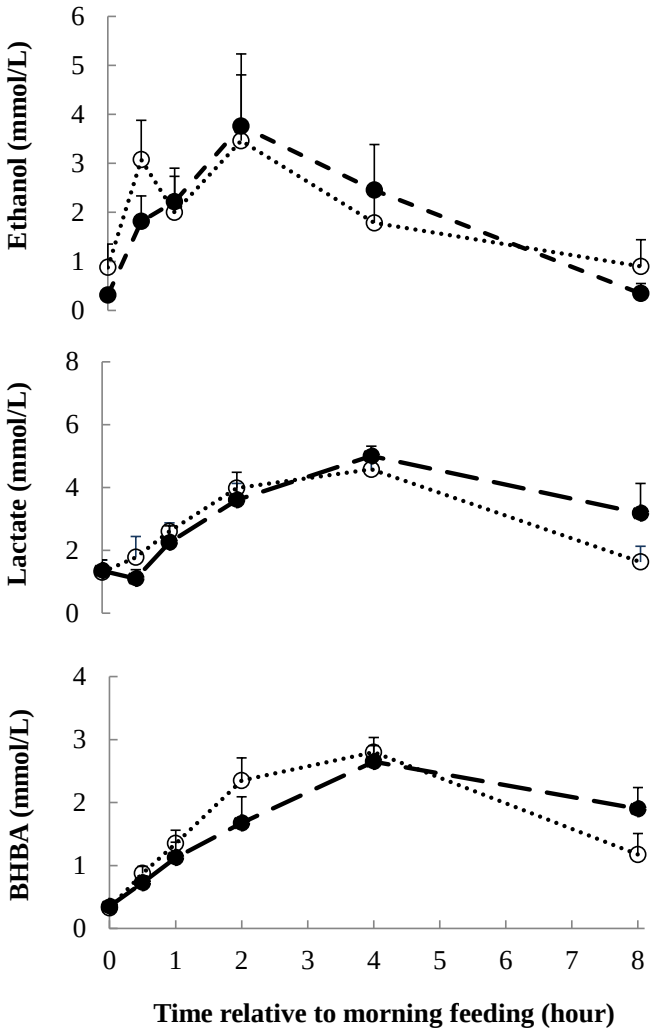


Figure 2.2.1: Time course changes of plasma Ethanol, Lactate and β-hydroxybutyrate (BHBA) of ewes fed on L-APS (○) or H-APS (●) treatment. The ethanol intake was calculated to be 37.1 and 50.1 g/d/head for L-APS and H-APS groups, respectively. Each data point is mean of 4 observations ± standard error of the means.

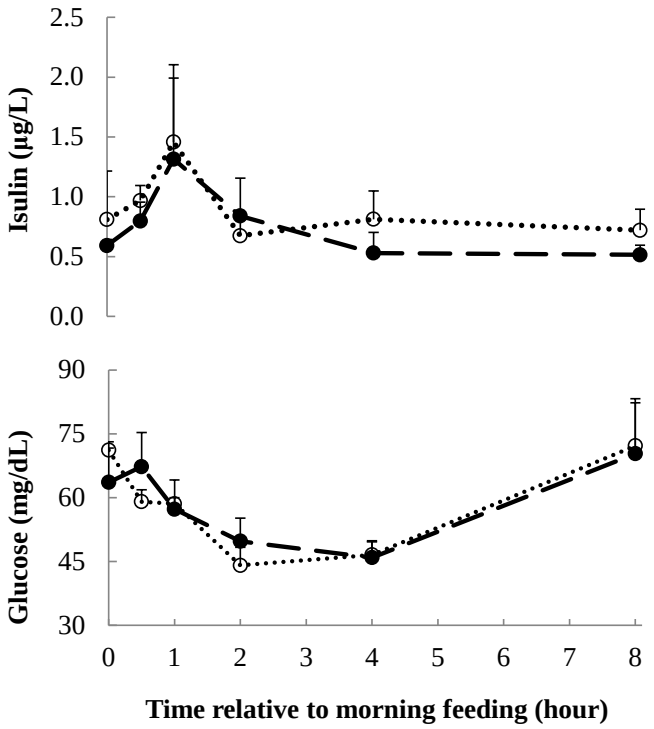


Figure 2.2.2: Time course changes of plasma Insulin and Glucose of ewes fed on L-APS (○) or H-APS (●) treatment. The ethanol intake was calculated to be 37.1 and 50.1 g/d/head for L-APS and H-APS groups, respectively. Each data point is mean of 4 observations ± standard error of the means.

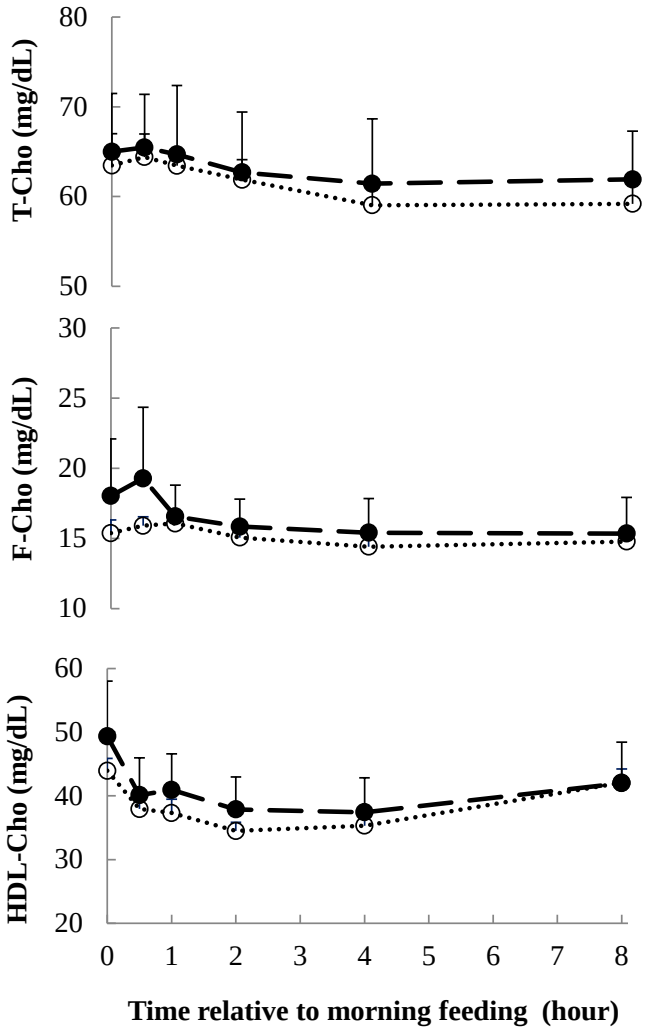


Figure 2.2.3: Time course changes of plasma Total-Cholesterol (T-Cho), Free-Cholesterol (F-Cho) and high density lipoprotein cholesterol (HDL-Cho) of ewes fed on L-APS (○) or H-APS (●) treatment. The ethanol intake was calculated to be 37.1 and 50.1 g/d/head for L-APS and H-APS groups, respectively. Each data point is mean of 4 observations ± standard error of the means.

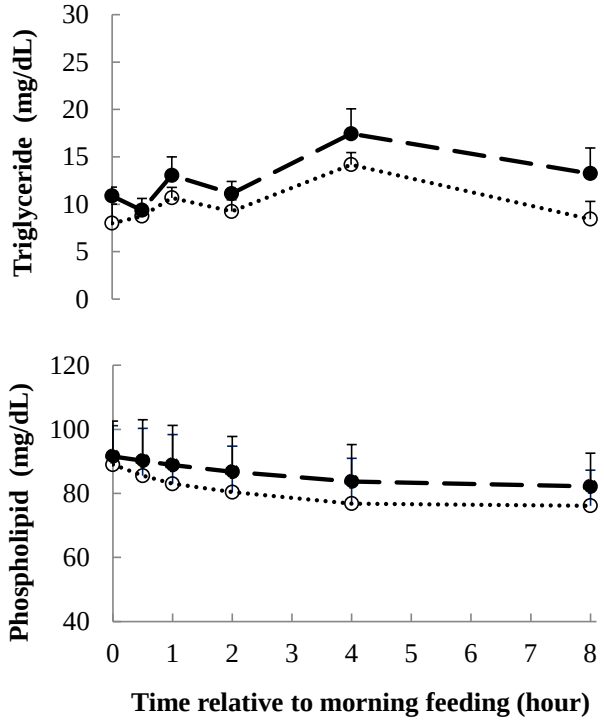


Figure 2.2.4: Time course changes of plasma Triglyceride and Phospholipid of ewes fed on L-APS (○) or H-APS (●) treatment. The ethanol intake was calculated to be 37.1 and 50.1 g/d/head for L-APS and H-APS groups, respectively. Each data point is mean of 4 observations ± standard error of the means.

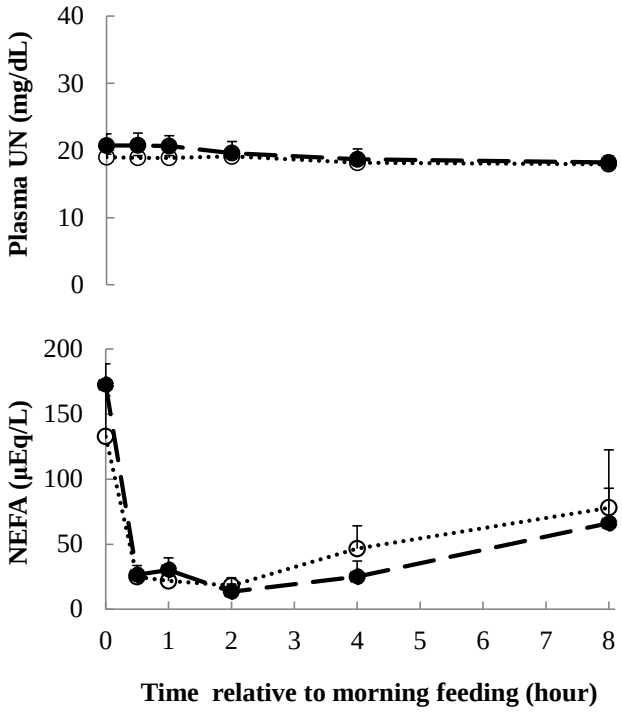


Figure 2.2.5: Time course changes of plasma urea nitrogen (UN) and non-esterified fatty acids (NEFA) of ewes fed on L-APS (○) or H-APS (●) treatment. The ethanol intake was calculated to be 37.1 and 50.1 g/d/head for L-APS and H-APS groups, respectively. Each data point is mean of 4 observations ± standard error of the means.

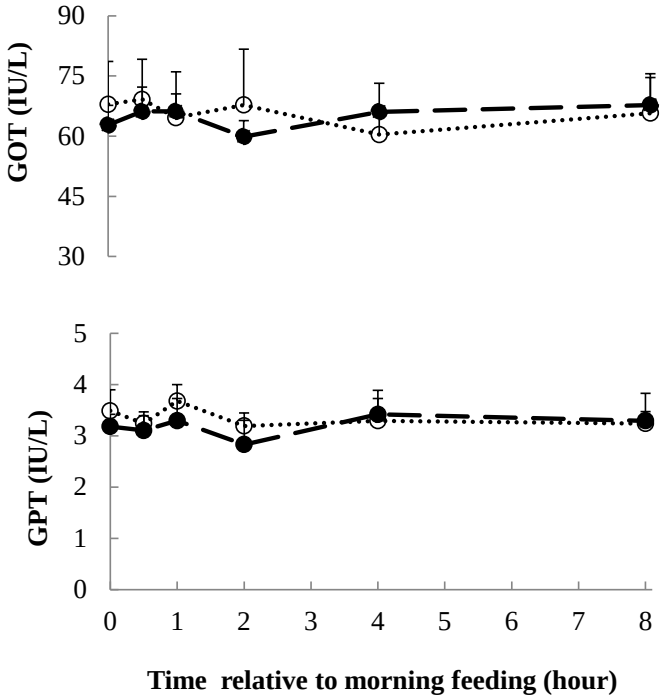


Figure 2.2.6: Time course changes of the enzyme activity of plasma glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) of ewes fed on L-APS (○) or H-APS (●) treatment. The ethanol intake was calculated to be 37.1 and 50.1 g/d/head for L-APS and H-APS groups, respectively. Each data point is mean of 4 observations ± standard error of the means.

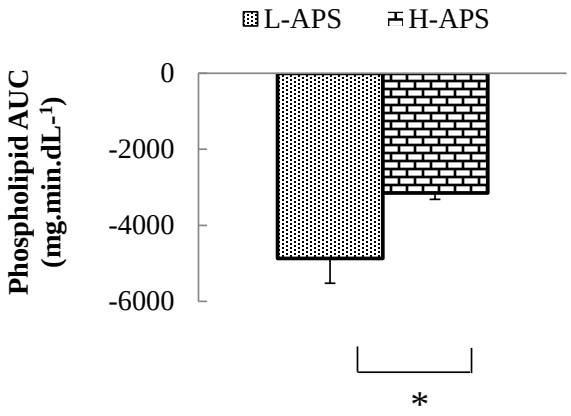


Figure 2.2.7: The area under the curve (AUC) of plasma phospholipid of ewes fed on L-APS (dotted bar) or H-APS (brick bar**)** treatment for 8 hours sampling windows. Each data point is the mean of 4 observations ± standard error of the means. The asterisk indicate a significant difference between treatments (*P < 0.05).

CHAPTER 3

Nutritive values of apple pomace silages containing different amounts of ethanol in Suffolk ewes

INTRODUCTION

It is increasingly important for countries to develop an efficient way to produce, utilize and improve the feed self-sufficiency rate for the domestically raised animal and reduce negative environmental impacts. Apple pomace (AP) is a nutritionally rich agro-industrial by-product. In the juice factory, waste from AP has increased rapidly in the recent years. Apple pomace contains 25-35% of the dry mass of apple (Gullón et al., 2007) which has high moisture, 200-250 g DM kg^{-1} (Taasoli and Kafilzadeh; 2008) with rich in fermentable carbohydrate and organic acid (Alibes et al., 1984). Apple pomace could be used as ruminant feed which is palatable, effective energy source and economical in apple growing area but poor in protein (Fontenot et al., 1977; Alibes et al., 1984; Gasa et al., 1992; Taasoli and Kafilzadeh, 2008). However, some studies were undertaken to utilize the AP with urea and other ingredients supplementation to balance the low protein and high moisture content (Rumsey et al., 1978; Alibes et al., 1984; Nikolić and Jovanović., 1986; Pirmohammadi, et al., 2006), but performance was not satisfactory. If AP would be ensiled with other feed ingredients to absorb the high moisture and balance the low protein content similar to a concentrate mixer, then that will be good quality silage and high amount of ethanol would become minimize. Although, limited researches are available about the nutritive value of AP and APS but information on the nutritive value of nutritional balanced fermented APS and its effect on blood biochemistry in sheep still unknown.

Therefore, the current study was undertaken to evaluate the fermentation characteristics, digestibility and nutritive value of APSs which were low ethanol contains APS (L-APS) and high ethanol containing APS (H-APS). After feeding of

nutritional balanced APSs, the effect on blood parameters of Suffolk shorn ewes were also investigated.

MATERIALS AND METHODS

All procedures involving animals in this study were approved by the Institutional Animal Care and Use Committee at Hirosaki University (A08023).

1. Animal, Experimental Design and Management

Three Suffolk shorn ewes (aged 11 months on average and weighing 51.1 ± 1.5 kg) were used in a 3 × 3 Latin square design over three 21 days periods including of initial 7 days for dietary adaptation and the last 7 days for sample collection. The ewes were randomly assigned to three dietary treatments: alfalfa hay cube and either L-APS or H-APS provided as half of TDN requirement for L-APS or H-APS diets and hay diet receiving group fulfil their TDN requirement from alfalfa hay cube. Daily allowance was offered as TDN requirement for 110% maintenance (Ministry of Agriculture, Forestry and Fisheries, 1996; Japan) in 2 equal meals at 10:00 and 18:00. The required TDN amount was adjusted based on their body weight (BW). BW was measured before the morning feed at the beginning of each period, at the end of first 7 days (adaptation period) and at the end of each period. The ewes were housed in individual pens with sawdust bedding in an indoor animal room during the feed adjustment period (first 7 days) and then moved to metabolic cages for next 14 days where the last 7 days was for sample collection. According to the adaptation periods BW, same feed allowance was offered for the last 14 days. Trace mineralized salt block and water were offered access throughout the experiment.

2. Apple pomace silage preparation

Fresh AP was obtained from an apple juice factory of the Farm village industry federation of Aomori prefectural agricultural cooperatives at Hirosaki, Japan. The L-APS and H-APSs were prepared with 70% fresh AP or fermented AP, 6% soybean

meal, 12% wheat bran and 12% beet pulp at an identical ingredient composition which was formulated to have similar nutrient contents to the concentrate (CP 15.5% and TDN 70%). Because remaining sugars in fresh AP is fermented rapidly and efficiently turned into ethanol under solid-state fermentation condition (Hang et al., 1981), fresh AP was stored alone in an anaerobic condition for 2 months to secure higher ethanol contents before ensiling of H-APS. For ensiling of L-APS, fresh AP within 2 days of production was used. The AP and all other ingredients were mixed thoroughly by a mechanical mixer and stuffed into plastic container, pressed sufficient to fill properly, topped with air-tight cover and ensiled for 2 months before use in the feeding experiment.

3. Collection of feed, feed refusals, urine and faeces samples

Urine and faeces were collected daily in the morning during the last 7 days. Urine was collected in the plastic pots which containing 50 ml of 6N H_2SO_4 solution to prevent nitrogen (N) loss. Total amount of daily excreted urine and faeces by each animal was measured, recorded and 10% well mixed sub-sample were stored at 4°C during the collection period. Last day of each period total excreted urine and faeces sub-sample were well mixed and again 10% sub-samples were taken for further analysis. If there had some residue of feeds, that was also collected daily in the morning during the collection period. At the end of each period, all feeds were sampled for chemical analysis and silages were also sampled for fermentation quality analysis.

4. Feed, feed refusals, urine and faeces samples analyses

Apple pomace silage extraction procedure and determination procedure of pH, VBN, organic acid and ethanol were same as described in the previous part (Chapter 2.1). All feed samples, feed refusal and faeces were dried in a forced-air oven at 60 °C for 48 hours, ground through a 1 mm screen using a Willey mill and kept in a plastic airtight container for chemical analyses. Dry matter (DM), crude protein (CP), crude fiber (CF), ether extract (EE), ash, neutral detergent fiber (NDF) and acid detergent

fiber (ADF) were determined by following the methods as described in the previous part (Chapter 2.1). Urinary nitrogen (N) content was determined by Kjeldahl method (AOAC1 1990). Chemical composition of experimental feeds ingredients are presented in the previous part (Table 2.2.2).

5. Blood samples collection and analytical methods

On the last day of each 21 day period, series of blood samples were obtained by jugular venipuncture at pre-feeding (0) and at 0.5, 1, 2 and 3 hours after the morning feed. On each occasion blood was collected into two 7 mL evacuated tube containing sodium heparin as anticoagulant, chilled on ice and centrifuged (3000 × g for 15 min at 4 °C) to harvest plasma sample. Plasma was aliquot and stored at -30 °C until analyzed for ethanol, lactate, β-hydroxybutyrate (BHBA) and blood metabolites [glucose, non-esterified fatty acids (NEFA), plasma urea nitrogen (UN), total cholesterol (T-Cho), free cholesterol (F-Cho), high density lipoprotein cholesterol (HDL-Cho), phospholipid and triglyceride]. Another plasma aliquot was stored at -80 °C until analysed for the ethanol-induced liver injury biochemical marker of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT). All plasma samples were assayed by the following procedures as described in the previous part (Chapter 2.1). The area upper or under the curve (AUC) for 3 hours of plasma parameters were calculated as an indicative of the changes in the parameters.

6. Calculation

Calculation procedure of hemicellulose, NFC, BWG and TDN intake were same as described in the chapter 2.1. Ethanol intake was calculated from APS intake and ethanol content of the APS. The digestibility of L-APS and H-APS feeds were calculated by difference according to Schneider and Flatt (1975). The ME values of the feeds were calculated using the following equation (Agricultural and Food Research council, 1993): ME (MJ/kg DM) = 0.016DOMD.

Where,

ME, metabolizable energy

MJ, mega joules

kg, kilo gram

DOMD, digestible organic matter as dry matter basis.

7. Statistical Analysis

Least significant difference (LSD) test of SPSS was used to compare the treatment means for chemical composition, fermentative products, apparent digestibility and nutritive values of feeds. Digestibility trial and plasma AUC data were analyzed as a replicated 3×3 Latin square using the general linear model (GLM) procedure of SPSS, with ewe, period and diet were included in the model. Serial data for blood parameters were analyzed with polynomial contrasts to determine the slop response of the time influence and their interaction with diet. Tukey test (SPSS) was used to identify differences ($P < 0.05$) between means. Relationship among the AUC responses for plasma measurements was analyzed by Pearson's correlation coefficient.

RESULTS

1. Organic acid and other fermentation profiles

The pH value, organic acids and others fermented product of AP and APSs are presented in Table 3.1. The pH value was identical in AP and FAP (4.01 and 4.08; respectively). However, pH values were slightly decreased during ensiling but pH levels were almost similar in both APSs. Fresh AP contained considerable amount of lactic acid (15 g kg^{-1} DM) which influenced multiplication (35 g kg^{-1} DM) of lactate during the ensiling in L-APS. Lactic acid concentration was rapidly increased (72.9 g kg^{-1} DM) in FAP during stored anaerobically for 2 months but lactate content was decreased during the ensiling of H-APS (36.0 g kg^{-1} DM). Fresh AP contained a small amount of acetic acid (5 g kg^{-1} DM) which was slightly increased in L-APS (15 g kg^{-1} DM). But FAP had a high degree of acidity (40.6 g kg^{-1} DM) which was minimized

during the ensiling of H-APS (23.6 g kg^{-1} DM). Fresh AP had negligible amount of ethanol (1.3 g kg^{-1} DM) and during ensiling ethanol content was increased (48.7 g kg^{-1} DM) in L-APS. Alcoholic fermented AP contained the largest amount of ethanol (242.5 g kg^{-1} DM) but that ethanol content was notably minimized (87.2 g kg^{-1} DM) by mixing with other ingredients of H-APS. Moreover, ethanol content of L-APS and H-APS were significantly remarkable. A negligible amount of VBN occurred in fresh AP (0.06 g kg^{-1} DM) which was slightly increased in L-APS (0.33 g kg^{-1} DM). A small amount of VBN occurred in alcoholic fermented AP (0.13 g kg^{-1} DM) which was slightly increased in H-APS (0.65 g kg^{-1} DM). The H-APS contained higher amount of moisture than that of L-APS. Bad smell and fungal growth were not found by the APSs during the experimental period.

2. Chemical composition of APSs

The chemical composition of APSs is shown in Table 3.2. High moisture containing fermented AP influenced the lower DM and OM content ($P < 0.01$ and $P < 0.05$) and higher CP, CF and ash content ($P < 0.05$) for H-APS feed. Fresh AP influenced the NFE ($P < 0.05$) content of L-APS. Ensiling process did not affect to change either the EE or ADF content of APSs. By using fermented AP for the preparation of H-APS, increased the NDF and hemicellulose content ($P < 0.01$). Fresh AP improved NFC in L-APS ($P < 0.01$).

3. Chemical composition of diets

The chemical composition of experimental diets is shown in Table 3.3. The hay diet contained higher ($P < 0.01$) DM content than that of the two APS diets. The H-APS diet contained lower ($P < 0.05$) DM content than that of L-APS diet. The OM content of hay diet was lower ($P < 0.01$) than that of APS diets and L-APS diet contained slightly higher ($P < 0.05$) OM content than that of H-APS diet. No notable difference occurred in CP content. Hay diet contained higher ($P < 0.01$) CF and lower ($P < 0.05$) EE contents than that of APS diets. However, CF and EE contents of L-APS

and H-APS diets did not differ significantly ($P > 0.05$) between each other. The highest ($P < 0.01$) crude ash was observed for hay diet and lower ($P < 0.05$) content was for L-APS diet. The L-APS diet contained higher ($P < 0.01$) NFE level than that of other two diets. The NFE content of H-APS diet was also higher ($P < 0.05$) than that of hay diet. Hay diet contained higher ($P < 0.01$) ADF and NDF content than that of L-APS and H-APS diets. No notable difference was observed in ADF content for APS diets but H-APS diet contained slightly higher ($P < 0.05$) NDF content than that of L-APS diet. The H-APS diet contained highest ($P < 0.01$) hemicellulose than that of other two diets. Besides, hemicellulose content of L-APS diet was significantly ($P < 0.05$) higher than that of the hay diet. The NFC level was higher ($P < 0.01$) in L-APS diet than that of other two diets and H-APS contained higher ($P < 0.05$) NFC than that of hay diet.

4. Apparent digestibility and nutritive value of diets

Apparent digestibility and nutritive value of different experimental diets are shown in Table 3.4. The apparent digestibility of DM, OM and CP were significantly lower ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively) in hay diet than those of APS diets. The digestibility of CP was slightly lower ($P < 0.05$) in L-APS diet than that of H-APS diet. The digestibility of EE, NFE, NDF and hemicellulose were significantly higher ($P < 0.05$, $P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively) in the both of APSs than those of hay diet. There was no significant difference between the L-APS diet and H-APS diet for the digestibility of DM, OM, EE, NFE, NDF and hemicellulose content. The CF and ADF digestibility did not differ significantly ($P > 0.05$) among the diets.

The digestible OM (DOM) was significantly higher ($P < 0.01$) for L-APS and H-APS diets than that of hay diet. But there was no significant difference between the L-APS and H-APS diets for DOM. The DCP was similar for all diets. The TDN content and metabolizable energy (ME) value were significantly higher ($P < 0.01$) in the APS diets than those of hay diet but no significant difference was observed between the L-APS and H-APS diets.

5. Apparent digestibility and nutritive value of L-APS and H-APS (by difference method)

Apparent digestibility and nutritive value of APSs which were calculated by difference method are shown in Table 3.5. There was no significant different between the L-APS and H-APS for digestibility of all nutrients. No significant result was observed between the L-APS and H-APS for DOM and DCP. There was no notable difference between the both APSs for TDN content and ME value also.

6. Nitrogen retention

Nitrogen retention of ewes fed different experimental diets is presented in Table 3.6. The ewes fed on APS diets showed lower ($P < 0.01$) nitrogen intake than the ewes fed on hay diet. The ewes fed on hay diet showed significantly higher ($P < 0.01$) faecal nitrogen excretion than the ewes fed on L-APS and H-APS diets. Besides, faecal nitrogen excretion of ewes fed on diet L-APS was slightly higher ($P < 0.05$) than that of the ewes fed on diet H-APS. The urinary nitrogen excretion was tended to higher in hay diet than that of APS diets, however, no significant difference were observed for L-APS and H-APS diets. Total nitrogen excretion was higher ($P < 0.05$) for the ewes fed on hay diet than that of the ewes fed on APS diets. However, no significant difference was detected between the L-APS and H-APS diets for total nitrogen excretion. No negative balance was observed in any of the ewes throughout the collection period. There was no significant difference among the diets for the percentage of nitrogen retention.

7. Performance traits of ewes fed different experimental diets

No health problems were observed in any of the ewes throughout the experimental periods. Performance traits of ewes are presented in Table 3.7. There was no significant ($P > 0.05$) difference among the diets for initial BW, final BW, BWG and TDN intake. The DM intake was higher ($P < 0.01$) in hay diet than that of APS diets but there was no significant difference between the L-APS and H-APS diets.

Ethanol intake was higher ($P < 0.05$) for the ewes fed on H-APS diet than that of L-APS diet whereas ewes on hay diet were assumed to receive any of alcohol.

8. Effect of feeding APSs on blood parameters

8.1 Time course changes of plasma parameters

Time course changes of plasma ethanol, lactate, BHBA and glucose in ewes fed different experimental diets are illustrated in Figure 3.1. After ingestion of APSs, plasma ethanol concentration were abruptly increased ($P < 0.01$) and H-APS diet showed higher value than that of L-APS diet and peaked at 0.5 hour and 1 hour after feeding for L-APS and H-APS; respectively. These increased concentrations for APS diets were not returned to the previous value until 3 hours after feeding and H-APS diet represented the remarkable value (3.68 mmol L^{-1}) than those of other diets. After ingestion, plasma lactate concentrations were increased ($P < 0.05$) in APS diets receiving ewes than that of hay diet and peaked at 2 hours after feeding for both APSs. That concentration for L-APS diet declined over the last 3rd hour after feeding but that was unchanged for H-APS diet receiving group. After ingestion of APSs, plasma BHBA concentrations were gradually increased ($P < 0.01$) over the first 3 hours after feeding for both APS diets. Postprandial hypoglycaemic tendency ($P = 0.06$) occurred in both APS diets receiving ewes and plasma glucose concentrations were declined over the first 2 hours after feeding for both APS diets. Glucose level was increasing very slowly in H-APS receiving ewes at 3 hours after the morning feed.

Time course changes of plasma T-Cho, F-Cho and HDL-Cho in ewes fed different experimental diets are illustrated in Figure 3.2. After feeding, the plasma concentration of T-Cho were increased ($P < 0.05$) in APS receiving groups than that of hay diet and peaked at 0.5 hour for both APS diets. After ingestion of APSs, F-Cho concentrations were always tended to increase ($P < 0.1$) in APS diets than that of hay diet. The peaked at 0.5 hour in all of groups and the highest concentration was observed in L-APS receiving group. Plasma HDL-Cho concentrations were higher for both APS diets however, no significant effect was detected among the diets.

Time course changes of plasma triglyceride and phospholipid in ewes fed different experimental diets are illustrated in Figure 3.3. After feeding, plasma triglyceride concentration was not influenced by diets among the dietary groups. After ingestion of APS diets, plasma phospholipid concentrations were tended to elevate ($P < 0.1$) and peaked at 0.5 hour for both APS diets receiving ewes.

Time course changes of plasma UN and NEFA in ewes fed different experimental diets are illustrated in Figure 3.4. Plasma UN concentrations for both APSs were slightly lower than that of hay diet however, no significant difference was detected. There was no significant difference among the dietary groups for plasma NEFA concentrations. No significant effect was also observed for the enzyme activity of plasma GOT and GPT (Figure 3.5).

8.2 Response of area upper or under curve (AUC)

The AUC of plasma parameters for 3 hours sampling windows are represent in Table 3.8. The AUCs of plasma ethanol were greater ($P < 0.01$) for APS diets than that of hay diet (Figure 3.6A) and the H-APS diet had greater ($P < 0.05$) AUC than that of L-APS diet. No significant difference was observed among the diets for the AUC of plasma lactate. The AUC of plasma BHBA in the both APS diets were greater ($P < 0.01$) than that of hay diet (Figure 3.6B). However, there was no significant difference between the L-APS and H-APS diets for the AUC of plasma BHBA. No significant differences were observed among the diets for the AUC of plasma glucose, T-Cho, F-Cho, HDL-Cho, triglyceride, phospholipid, NEFA, plasma UN, GOT and GPT.

8.3 Correlation among AUC responses of plasma measurements

The correlation coefficient among the AUCs of plasma ethanol, lactate, BHBA, plasma metabolites, GOT and GPT for 0-3 hours sampling windows are presented in Table 3.9. Plasma ethanol AUC was positively correlated ($P < 0.05$) with the AUCs of plasma lactate, triglyceride and GOT. Plasma ethanol AUC was negatively correlated ($P < 0.05$) with the AUC of plasma urea nitrogen. The lactate AUC was positively

correlated with the AUCs of F-Cho, triglyceride and GOT ($P < 0.05$, $P < 0.01$, $P < 0.05$, respectively). Plasma lactate AUC was negatively correlated ($P < 0$ .01) with that of the plasma urea nitrogen. The plasma BHBA AUC was negatively correlated ($P < 0$ .01) with that of the plasma glucose.

DISCUSSION

The higher amount of moisture and lower CP content of AP are in anxiety with earlier reporters (Alibes et al., 1984; Pirmohammadi et al., 2006; Taasoli and Kafilzadeh; 2008). Therefore, it was required to adjust the high moisture and improve nutritional balance of AP with addition of other ingredients and for better understanding of the fermentation products with nutrient efficiency of balanced APSs. In fact in the present study, higher moisture content was observed in fermented AP in comparison with APSs that blended with other ingredients to improved nutritional balance of AP after 2 months storage under the similar anaerobic condition. The spongy like nature of AP fiber, with its “easy-to-absorbs” and easy-to-release” moisture capacity may be mostly responsible for this. Moreover, high fermentative sugar (Alibes et al., 1984) and more susceptibility to enzymatic hydrolysis (Gullón et al., 2007) of AP leads to a rapid production of ethanol and lactic acid because there is enough soluble carbohydrate available for sugar fermentation by the action of lactic acid bacteria and yeast. The alcoholic AP was identified as the largest ethanol content (242.5 g kg^{-1}) than that of in a single APS (173 g kg^{-1}) reported by Alibes et al. (1984). It’s indicated that drained fluid of H-APS were particularly rich in alcohol. By using fermented AP for preparation of balanced H-APS, we succeed to increase its ethanol content by 36% with compared to that of L-APS which indicate that various concentrations of alcohol in silages are depending on the fermentation profile (Kristensen et al., 2010). Ethanol content of APSs used in this experiment were more than 1.5 to 2.6 times higher than that of RS-APS (Chapter 2.1), 3 to 4 times higher than that of corn silage (McDonald et al., 1991; Raun and Kristensen, 2010), 4 to 6 times higher than that of grass silage (Lawrence et al., 2011) and 4 to 5 times higher than that of ensiled TMR silages

(Cummins et al., 2009). H-APS contained about 1.4 times higher ethanol than that of sugarcane silage (Daniel et al., 2013). APSs presented considerable amount of lactic acid, small amount of acetic acid, especially no butyric acid and lower pH level, indicating a good fermentation quality of them (McDonald et al., 1973; Pirmohammadi et al., 2006). Probably, due to the lower pH and higher lactic acid content improved the silage storage quality and protects the fungal growth.

The main losses in low DM are associated with the ensiling process and effluent loss (McDonald et al., 2011). The lower DM and OM content of H-APS in comparison with L-APS was likely due to rich in drained fluid as well as rapidly degradability of water-soluble carbohydrates. However, DM content of APSs were higher than that of other results (14.5-21.5%, 17.2-28.4%, 28.4% and 24.7% reported by Alibes et al., 1984; Nikolić and Jovanović, 1986; Pirmohammadi et al., 2006, and Taasoli and Kafilzadeh, 2008, respectively). Dried ingredients may improve the DM content in APSs. Pirmohammadi et al. (2006) who worked with mixed ensiled AP (1 tonne AP + 100 kg wheat straw + 5 kg urea, as fresh basis) and observed slightly lower OM content (92.5 g kg^{-1} DM) than that of in this study. The ensiling proteolysis process increases in the proportion of ammonium nitrogen and free ∝-amino acid nitrogen in the silage compared with the original material (McDonald et al., 2011) which was the reason of higher CP content in H-APS compared with L-APS. Hang et al. (1981) reported that yeast fermentation increase the amount of protein in AP by over 50% whereas in this study was 67% for fermented AP and was 6.4% higher in H-APS compare with L-APS. The APSs were formulated to have similar nutrient contents to the commercial concentrate, which has led to increase in CP content (17 - 18%) than that of other results (6.7-6.8%, 7.2% and 6.4%, on DM basis reported by Alibes et al., 1984; Pirmohammadi et al., 2006 and Taasoli and Kafilzadeh, 2008, respectively). The CF, ash, NDF and hemicellulose content were higher in H-APS due to the highly fermentative sugar in fermented AP. The ADF content of APSs were lower than that of other results (34.8-35.3%, 46.0% and 25.7% on DM basis reported by Alibes et al., 1984; Pirmohammadi et al., 2006 and Taasoli and Kafilzadeh, 2008, respectively).

However, Alibes et al. (1984) and Taasoli and Kafilzadeh (2008) reported the similar value for NDF of APS (41.3-42.7% and 38.6% on DM basis, respectively). During fermentation, even a small amount of water soluble carbohydrate degraded by lactic acid bacteria could decrease NFE and NFC levels (Cai et al., 2001, 2003). Hay diet contained higher amount of DM, CF, ash, ADF and NDF content and lower amount of OM, EE, NFE, hemicellulose and NFC content compare with the APSs diets due to higher amount of dry mass in alfalfa hay cube. This is also an indication of high percentage of AP material in the APSs.

The digestibility of DM, OM, CP, CF, EE, NFE, NDF and hemicellulose were higher in the ewes fed APSs than those of alfalfa hay diet receiving ewes. Ahn et al. (2002) worked with AP (60% AP + 30% rice bran + 10% concentrate) and reported that slightly lower DM values (71.6%) for Korean goat than those of APSs in his study (77.9% to 79.5%). Method of silage making, ensiling condition and type of feed ingredients as the absorbent, may have a great impact on the quality of silage and hence the amount that is consumed by animal. Alibes et al. (1984) reported that when diets containing high amount of APS then the OM digestibility for APS diets were 70.1% to 77.7% and estimated OM digestibility for APSs were 74.4% to 80.4%. These estimated values are correspond very well to the fact that ewes received 70% fresh or fermented AP containing L-APS/H-APSs and the OM digestibility for APSs were obtained 79.6% to 80.9%, however APSs diets showed slightly lower values (67.9% to 68.9%) in the present study. Probably the higher amount of hay cube influences the lower OM digestibility for APS diets. However, Taasoli and Kafilzadeh (2008) found slightly lower OM digestibility values 69.9% and 71.8% for ensiled and dried AP compared to these findings. Fermented APSs were a replacer of concentrate for ruminants, indeed could increase the estimated apparent CP digestibility of L-APS (74.6%) and H-APS (75.7%) and that level was slightly decreased in the APS diets (73.2% and 73.6% for L-APS and H-APS diets, respectively). However, APS diet having higher digestible CP than that of hay diet (72.3%) and fermented AP influenced slightly higher CP digestibility for H-APS diet than that of LAPS diet. On the other hand, Alibes et al.

(1984) found lower estimated apparent CP digestibility for APSs (18.0% to 45.3%) and APS diets also (44.9% to 63.4%). Alibes et al. (1984) also reported similar values for estimated apparent CF digestibility (82.2% to 86.2%) of APSs and slightly higher values (63.1% to 83.5%) for APS diets. In addition, fermented APSs had a higher amount of lactic acid which was extracted out and mixed with EE, resulted as a higher level of EE digestibility in APSs and APS diets. Cellulose digestibility was higher in APSs diets than that of hay diet, probably hydrogen availability was increased by fermented APS ethanol which influenced the cellulose digestion. This result is an agreement with Chalupa et al. (1964), who found that under in-vitro condition ethanol increase cellulose digestion. The special nature of AP improved the digestible OM value (62.6% and 61.6% for L-APS and H-APS diets, respectively) than that of alfalfa hay diet (53.3%) and the digestible OM value for APSs were obtained 74.9% to 76.5%. However, Pirmohammadi et al. (2006) reported lower digestible OM value (57.5%) than that in the present study. This inconsistent result may be due to differences in variety, environment conditions, concentration of cell wall, and technological difference in the juice extraction processes or losses of valuable volatile constituents during dehydration. Daily intake of digestible CP (DCP) were 141.4, 121.8 and 125.8 g in hay diet, L-APS diet and H-APS diet, respectively in this experiment which were enough to meet the daily requirements (92 g) of 50 kg ewe lambs (Ministry of Agriculture, Forestry and Fisheries, 1996; Japan). The higher values for digestibility probably contributed to higher TDN value in APSs with fresh AP and fermented AP compared to the alfalfa hay cube. Givens and Barber (1987) worked with fresh AP and Pirmohammadi et al. (2006) worked with ensiled AP (1 tonne AP + 100 kg wheat straw + 5 kg urea) and observed lower values for ME (8.7 and 9.0 MJ/kg DM, respectively) than those in this study (12.0-12.2 MJ/kg DM). Such difference might be justified by the contrasting processing methods and additional ingredients. We have not found any significant differences between the L-APS and H-APS for nutrient digestibility and nutritive values. Pirmohammadi et al. (2006) also reported the ME value for maize silages (10.3 MJ/kg DM) which was lower than those in this study. Such difference

indicate that this nutritionally balance fermented APSs are capable to overcome the constrain of low protein content in AP and will able to fulfil the protein requirement of ruminants. We have formulated daily ration to meet the daily 110% maintenance TDN requirement of growing ewe lambs (Ministry of Agriculture, Forestry and Fisheries, 1996; Japan) but did not follow DM requirement because APS had low DM value. Under this condition, higher DM containing alfalfa hay cube intake increased the higher nitrogen intake in the hay diet. In addition, APSs had more nutrients, DCP, TDN and ME, and resulted in less nitrogen excretion in faeces and urine. Nitrogen retention efficiency was also higher in APS diets but the values were non-significant. Under such energy-sufficient condition in APSs, nitrogen utilization was higher in APSs receiving ewes which may have stimulated growth. Although, BWG was not significant, APSs receiving ewes had 41% higher growth rate than that of hay diet. Any of performance traits were not affected by a difference in ethanol intake (42%) between L-APS and H-APS diets in this experiment. Therefore, ingestion does of ethanol (~15 g/d) from APSs in this study would not have any harmful effect on performance of growing ewes.

After ingestion of APSs, plasma ethanol, lactate and BHBA concentrations were increased and concentrations were not returned to pre-feeding levels at 3 hours after morning feed. Such increased tendency has been reported by Kristensen et al. (2007) and these findings were also similar with our previous findings of RS-APS feeding in chapter 2.1. The higher level of dietary ethanol ingestion significantly influenced the plasma ethanol concentration which was remarkable at peak level for L-APS and H-APS (3.64 and 7.10 mmol/L, respectively). Plasma ethanol concentrations were not returned to pre-feeding levels at 3 hours after morning feeding and H-APS showed higher ethanol concentration (3.68 mmol/L) than that of L-APS at peak level. This corresponds with hypothesis the feeding dietary ethanol led to accumulation in plasma ethanol (Jean-Blain et al., 1992; Kristensen et al., 2007; Kondo et al., 2010, 2011; Raun and Kristensen, 2011). Jean-Blain et al. (1992) who worked with kinetic study of infused ethanol assumed that daily ethanol intake (0.2 to 1 g kg^{-1} BW) can be metabolized by rumen micro flora and enzymatic system of the host and plasma ethanol

level remains below 0.25 g/L. These estimates are exceeded the level in present study because daily ethanol intake were 0.2 and 0.3 g kg^{-1} BW and the highest plasma ethanol levels was 0.17 - 0.33 g/L. Excess amounts of readily fermented carbohydrate in ruminants diets is associated with the higher amount of lactate accumulation (Owens et al., 1998) and thus cause increased plasma lactate in ewes fed on APS diets for this experiment. Abrupt glucose supply from silage has often been shown to down regulate hepatic glucose production (Kristensen et al., 2007; Plaizer et al., 2005) in ruminants. Moreover, ethanol inhibits hepatic gluconeogenesis from non-carbohydrate sources such as lactate, glycerol, some amino acids and other substrates (Krebs et al., 1969) which is the cause of postprandial hypoglycemia in sheep fed on APS diets (Kondo et al., 2010; 2011). We also observed the similar hypoglycaemic condition after feeding of APSs in this experiment. Heitmann et al. (1987) reported that the multiparous ewes and high lactating dairy cow walk a fine line between glucose underproduction and ketone body overproduction. This relation is corresponding very well to the results of highly negative correlation between the AUC of plasma glucose and BHBA which indicating ethanol inhibited gluconeogenesis and resultant in postprandial hypoglycemia. Veenhuizen et al. (1991) also reported that hepatic in vitro gluconeogenic capacity decreased significantly for ketosis cow and at kitotic stage plasma BHBA increased up to 8.4 fold by using feed restriction plus dietary 1,3-butanediol. In the fact the sheep consumed APSs ethanol and plasma BHBA increased dramatically up to 9 and 8 fold at peaked hour for L-APS and H-APS, respectively which indicated that ketosis occurred in the ewes of this experiment. Holtenius and Holtenius (1996) reported that ketone bodies inhibit protein degradation and thereby gluconeogenesis and also are able to spare glucose by inhibiting glucose utilization and also inhibit lipolysis. In fact the opposite is true, fatty acid synthesis is increased and there is an increase in triglyceride production. Consistent the negative correlation of plasma UN with ethanol and lactate, negative correlation of BHBA with glucose and positive correlation of triglyceride with ethanol and lactate are strongly supporting these notions. Heitmann et al. (1987) reported that ketone body utilization is

accelerated in the neonate because of the high fat content of maternal milk. This concept is correspond very well to the fact that higher dietary fat contained in APSs influence the plasma lipid concentration. Therefore, we speculated that ethanol ingestion suppressed gluconeogenesis, increased lactate, hypoglycemia occurred and BHBA would also be generated from fatty acids.

Based on silages characteristics, it would be suggested that AP and fermented AP can be used for APS preparation in combination with other ingredients and inclusion levels of AP up to 70% for both state. Therefore, it may be concluded that AP can be well preserved by ethanol fermentation and nutritionally balanced APS can be used as a replacer of concentrate feed for ruminants in combination with hay cube or dry roughage up to 50% of the TDN requirement and ewes are capable to use 15.1 g/d ethanol intake without any clinical sign of adverse effect. Examination of responses in blood parameters after feeding of APS ethanol revealed increased plasma ethanol, lactate, BHBA and lipid components and tended to decrease glucose levels. The preparation of APSs with grains by products seems to be promising, as it prevents the loss of drain fluid that are especially rich in nutrients and contains moderate concentration of fermentation end products, which is a fact of importance as far as ethanol is concerned.

SUMMARY

The experiment was carried out to assess the nutritive value of apple pomace silages (APSs) and their effect on blood parameters in Suffolk ewes. Three ewes were used in 3×3 Latin square design over three period assigned with three dietary treatments: hay diet, low ethanol containing APS (L-APS, 48.7 g kg^{-1} DM ethanol) diet and high ethanol containing APS (H-APS, 87.2 g kg^{-1} DM ethanol) diet. Alfalfa hay cube and the L-APS or H-APS provide half of 110% TDN requirement for L-APS diet and H-APS diet, respectively and hay diet receiving group fulfil their 110% TDN requirement from alfalfa hay cube. On the last 7 days of each 21 days period, feaces, urine and feed refusal were collected and the last day of each period blood samples

were also collected before and after the morning feed for 3 hours. All plasma samples were assayed for ethanol, lactate, β-hydroxybutyrate (BHBA), glucose, non-esterified fatty acids (NEFA), plasma urea nitrogen (UN), total cholesterol (T-Cho), free cholesterol (F-Cho), high density lipoprotein cholesterol (HDL-Cho), phospholipid, triglyceride and glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT). The area upper or under the curve (AUC) for 3 hours and correlation among the AUCs responses were calculated.

The apparent digestibility of DM, OM, CP, CF, EE, NFE, NDF, and hemicellulose were higher ($P < 0.05$, $P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) in APS diets than those of hay diet and CP digestibility was lower in L-APS diet than that of H-APS diet. But there was no significant difference between the L-APS and H-APS diets for digestibility of others nutrient. Digestible OM, total digestible nutrient and ME values were higher in APS diets. But no difference was observed among the diets for the digestible CP. There was no significant difference in nutrient digestibility of all items and TDN content between the L-APS and H-APS.

Nitrogen intake and faecal nitrogen excretion were higher ($P < 0.01$) in ewes fed hay diet. Total nitrogen excretion was also higher ($P < 0.05$) in hay diet than that of APS diets. Ethanol intake was higher ($P < 0.05$) in ewes fed H-APS. After feeding, plasma ethanol concentration was significantly increased ($P < 0.01$) in APS diets and H-APS showed higher ethanol concentration than that of L-APS. The concentration of postprandial plasma lactate, BHBA and T-Cho were increased ($P < 0.05$, $P < 0.01$ and $P <0.05$, respectively) in ewes fed APS diets. After feeding, postprandial hypoglycemic tendency ($P < 0.1$) occurred in APS receiving ewes. Plasma concentration of F-Cho and phospholipid were tended ($P < 0.1$) to increase in APS receiving groups than those of hay diet group. The AUC of plasma ethanol was greater ($P < 0.01$) in APS diets than that of hay diet and L-APS diet had smaller ($P < 0.01$) AUC than that of H-APS diet. The AUC of plasma BHBA in the both APS diets were greater ($P < 0.01$) than that of

hay diet. However, there was no significant difference between the L-APS and H-APS diets for the AUC of plasma BHBA.

Plasma ethanol AUC was positively correlated ($P < 0.05$) with the AUCs of plasma lactate, triglyceride and GOT, and negatively correlated ($P < 0.05$) with the AUCs of plasma UN. The plasma lactate AUC was positively correlated ($P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively) with the AUC of F-Cho, triglyceride and GOT, and negatively correlated ($P < 0.01$) with that of plasma UN. The plasma BHBA AUC was negatively correlated ($P < 0.01$) with that of glucose. According to the consistent increase in plasma ethanol, lactate, BHBA, hypoglycaemic tendency, positive correlation of ethanol with lactate and negative correlation of BHBA with glucose, it would be concluded that postprandial hypoglycaemic tendency after APS feeding is due to the ingestion of ethanol which influence the hyperketonemia through suppression of gluconeogenesis. Based on the overall findings, it would be concluded that AP can be well preserved by ethanol fermentation and nutritionally balanced APS can be used as a replacer of concentrate feed for ruminants in combination with hay cube or dry roughage up to 50% of the TDN requirement. Therefore, ingestion does of ethanol (~15.1 g/d) from APSs in this study would not cause any harmful influence on performance of ewes.

Table 3.1: The pH value, organic acids and others fermented product of apple pomace and apple pomace silages.

Item	Fresh AP[1]	Fermented AP[2]	L-APS	H-APS
pH	4.01	4.08	3.85 ± 0.0	3.86 ± 0.2
Organic acids and other fermented products (g kg $^{-1}$ DM)				
Lactic acid	15.3	72.9	35.0 ± 10.0	36.0 ± 22.6
Acetic acid	5.0	40.6	15.0 ± 2.5	23.6 ± 15.0
Iso-butyric acid	0.9	ND	ND	ND
Butyric acid	ND	ND	ND	ND
Propionic acid	ND	ND	ND	ND
Total VFA[3]	5.9	40.6	15.0 ± 2.5	23.6 ± 15.0
Ethanol	1.3	242.5	48.7^{a} ± 5.1	87.2^{b} ± 14.9
VBN in TN	0.06	0.13	0.33 ± 0.1	0.65 ± 0.3
Moisture	767.0	856.9	633.8 ^{a} ± 6.9	663.1^{b} ± 5.6

[1]Fresh apple pomace within 2 days of production was used.

[2]Fermented AP was stored fresh AP for 2 months in an anaerobic condition.

[3]Total VFA = (Acetic acid + Iso-butyric acid + Butyric acid + Propionic acid).

[a,b]Different letters indicate significant difference among the treatments (at 5% level).

AP, apple pomace; L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; DM, dry matter; ND, not detectable; VFA, volatile fatty acid; VBN, volatile basic nitrogen; TN, total nitrogen.

Table 3.2: Chemical composition of low ethanol containing apple pomace silage and high ethanol containing apple pomace silage.

Item	L-APS	H-APS
Dry matter	37.7 ± 0.1	34.6 ± 0.2
Nutrient Composition (%, DM basis)		
Organic matter	94.5 ± 0.3	94.1 ± 0.1
Crude protein	17.2 ± 0.3	18.3 ± 0.4
Crude fiber	12.9 ± 0.3	14.2 ± 0.5
Crude ash	5.5 ± 0.3	5.9 ± 0.1
Ether extract	3.3 ± 0.4	3.5 ± 0.3
NFE	61.1 ± 0.8	58.1 ± 0.4
ADF	20.7 ± 0.2	20.8 ± 0.4
NDF	38.6 ± 0.2	42.3 ± 0.2
Hemicellulose	17.9 ± 0.4	21.5 ± 0.4
NFC[1]	35.4 ± 0.9	30.0 ± 0.6

[1]NFC = (100 - CP - EE -NDF - Ash).

L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; DM, dry matter; NFE, nitrogen free extract; ADF, acid detergent fibre; NDF, neutral detergent fibre; NFC, non-fibrous carbohydrate.

Table 3.3 Chemical composition of experimental diets[1]

Item	Hay diet	L-APS diet	H-APS diet	P value
Dry matter	$92.1^{c} \pm 0.6$	$58.2^{b} \pm 0.3$	$56.3^{a} \pm 0.2$	0.001
Nutrients Composition (%, DM basis)				
Organic matter	$88.5^{a} \pm 0.2$	$90.9^{c} \pm 0.1$	$90.6^{b} \pm 0.1$	0.001
Crude protein	17.1 ± 1.0	17.1 ± 0.5	17.5 ± 0.6	0.333
Crude fiber	$27.8^{b} \pm 0.7$	$21.8^{a} \pm 0.5$	$22.6^{a} \pm 0.2$	0.001
Ether extract	$1.8^{a} \pm 0.2$	$2.4^{b} \pm 0.2$	$2.4^{b} \pm 0.2$	0.032
Crude ash	$11.5^{c} \pm 0.2$	$9.1^{a} \pm 0.1$	$9.4^{b} \pm 0.1$	0.001
NFE	$41.8^{a} \pm 0.8$	$49.6^{c} \pm 0.2$	$48.0^{b} \pm 0.6$	0.007
ADF	$31.6^{b} \pm 0.4$	$27.3^{a} \pm 0.2$	$27.5^{a} \pm 0.3$	0.002
NDF	$46.4^{c} \pm 0.8$	$43.3^{a} \pm 0.5$	$44.9^{b} \pm 0.5$	0.004
Hemicellulose	$14.8^{a} \pm 0.9$	$16.0^{b} \pm 0.5$	$17.4^{c} \pm 0.7$	0.008
NFC[2]	$23.2^{a} \pm 0.6$	$28.1^{c} \pm 0.4$	$25.8^{b} \pm 0.6$	0.005

[1]Values are means with SD of three ewes per treatment.

[2]NFC = (100 - CP - EE - NDF - Ash).

[a,b,c]Different letters indicate significant difference among the treatments (at 5% level).

L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; DM, dry matter; NFE, nitrogen free extract; ADF, acid detergent fibre; NDF, neutral detergent fibre; NFC, non-fibrous carbohydrate, SD, stander deviation.

Table 3.4: Apparent digestibility and nutritive value of different experimental diets[1]

Item	Hay diet	LAPS diet	HAPS diet	P value
Apparent digestibility of nutrients (%)				
Dry matter	58.1[a] ± 0.9	66.7[b] ± 1.9	65.6[b] ± 0.2	0.010
Organic matter	60.2[a] ± 0.5	68.9[b] ± 1.9	67.9[b] ± 0.2	0.008
Crude protein	72.3[a] ± 2.0	73.2[b] ± 1.6	73.6[c] ± 1.7	0.003
Crude fiber	45.1 ± 1.0	54.3 ± 4.5	54.2 ± 1.9	0.043
Ether extract	28.3[a] ± 7.5	47.1[b] ± 1.5	45.9[b] ± 2.6	0.019
NFE	66.7[a] ± 0.9	74.8[b] ± 1.2	73.4[b] ± 0.8	0.010
ADF	42.5 ± 1.1	49.2 ± 3.7	48.0 ± 1.7	0.139
NDF	44.4[a] ± 1.8	53.1[b] ± 2.9	53.7[b] ± 0.8	0.018
Hemicellulose	48.2[a] ± 5.3	59.7[b] ± 3.6	62.7[b] ± 4.3	0.002
Nutritive value (%, DM basis)				
DOM	53.3 [a] ± 0.4	62.6 [b] ± 1.6	61.6 [b] ± 0.1	0.006
DCP	12.4 ± 1.0	12.5 ± 0.6	12.9 ± 0.7	0.158
TDN	54.0 [a] ± 0.5	64.0 [b] ± 1.7	63.0 [b] ± 0.3	0.004
ME (MJ/kg DM)	8.5 [a] ± 0.1	10.0[b] ± 0.7	9.9[b] ± 0.2	0.006

[1]Values are means with SD of three ewes per treatment.

[a,b,c]Different letters indicate significant difference among the treatments (at 5% level).

L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; DM, dry matter; NFE, nitrogen free extract; ADF, acid detergent fibre; NDF, neutral detergent fibre; DOM, digestible organic matter; DCP, digestible crude protein; TDN, total digestible nutrient; ME, metabolizable energy; MJ, mega joules; kg, kilo gram; SD, stander deviation.

Table 3.5: Apparent digestibility and nutritive value of L-APS and H-APS (Estimated by difference).

Item	L-APS	H-APS	P value
Apparently digestibility of feed (%)			
Dry matter	79.5 ± 5.0	77.9 ± 1.3	0.603
Organic matter	80.9 ± 4.3	79.6 ± 1.4	0.656
Crude protein	74.6 ± 6.1	75.7 ± 5.9	0.844
Crude fiber	83.5 ± 21.2	83.0 ± 8.4	0.969
Ether extract	63.2 ± 7.4	61.2 ± 9.5	0.792
NFE	83.1 ± 1.4	81.2 ± 1.5	0.170
ADF	64.3 ± 11.8	61.5 ± 3.4	0.714
NDF	68.8 ± 10.7	70.4 ± 5.5	0.825
Hemicellulose	74.2 ± 13.1	79.2 ± 11.4	0.642
Nutritive value of feeds (%, DM basis)			
DOM	76.5 ± 4.1	74.9 ± 1.2	0.563
DCP	12.9 ± 0.9	13.9 ± 1.2	0.296
TDN	79.1 ± 4.1	77.6 ± 1.4	0.596
ME (MJ/kg DM)	12.2 ± 0.7	12.0 ± 0.2	0.562

L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; DM, dry matter; NFE, nitrogen free extract; ADF, acid detergent fibre; NDF, neutral detergent fibre; DOM, digestible organic matter; DCP, digestible crude protein; TDN, total digestible nutrient; ME, metabolizable energy; MJ, mega joules; kg, kilo gram; SD, stander deviation.

Table 3.6: Nitrogen retention of ewes fed different experimental diets[1]

Item	Hay diet	L-APS diet	H-APS diet	P value
Nitrogen intake (g/d)	$31.0^{b} \pm 2.3$	$26.0^{a} \pm 0.6$	$25.5^{a} \pm 1.3$	0.002
Nitrogen in faeces (g/d)	$8.6^{c} \pm 0.4$	$7.0^{b} \pm 0.4$	$6.7^{a} \pm 0.1$	0.001
Nitrogen in urine (g/d)	17.2 ± 2.9	12.6 ± 0.8	13.5 ± 1.6	0.056
Total nitrogen excretion (g/d)	$25.7^{b} \pm 3.2$	$19.6^{a} \pm 0.4$	$20.2^{a} \pm 1.6$	0.029
Nitrogen retention (%)	16.5 ± 14.1	24.5 ± 3.0	20.4 ± 7.9	0.163

[1]Values are means with SD of three ewes per treatment.

[a,b,c]Different letters indicate significant difference among the treatments (at 5% level).

L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; %, percent; g/d, gm per day; SD, stander deviation.

Table 3.7: Body weights and Performance traits of ewes[1] fed different experimental diets[1].

Item	Hay diet	L-APS diet	H-APS diet	P value
Initial BW(kg)	51.3 ± 2.0	51.3 ± 1.7	50.7 ± 1.5	0.239
Final BW(kg)	51.7 ± 2.8	52.0 ± 2.8	51.4 ± 1.6	0.649
BWG (g/d)	19.4 ± 64.5	33.0 ± 69.3	33.0 ± 10.6	0.926
DM intake (g/d)	1136^{b} ± 59.5	948^{a} ± 32.1	908^{a} ± 21.9	0.008
TDN intake (g/d)	574 ± 21.0	573 ± 15.1	564 ± 19.4	0.357
Ethanol intake[2] (g/d)	0.0^{a} ± 0.0	10.6^{ab} ± 2.2	15.1^{b} ± 2.6	0.040

[1]Values are means with SD of three ewes per treatment.

[a,b,c]Different letters indicate significant difference among the treatments (at 5% level).

[2]Ethanol intake was calculated from the APS intake and ethanol content of the APS.

L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; BW, body weight; BWG, body weight gain; DM, dry matter; TDN, total digestible nutrient; g/d, gm per day; SD, stander deviation.

Table 3.8: The response of area upper or under curve of plasma parameters for 3 hours sampling windows of ewes fed different experimental diets[1].

Item	Hay diet	L-APS diet	H-APS diet	P- value
Ethanol (mmol.min.L^{-1})	-4[a] ± 20	362.8[b] ± 88	985.2[c] ± 130	0.003
Lactate (mmol.min.L^{-1})	-6 ± 5 4	94 ± 3 27	421 ± 88	0.202
BHBA(mmol.min.L^{-1})	30[a] ± 21	177[b] ± 14	149[b] ± 24	0.008
Glucose (mg.min.dL^{-1})	285 ± 472	2711 ± 1030	2630 ± 877	0.132
T-Cho (mg.min.dL^{-1})	-293 ± 59	-160 ± 333	-14 ± 592	0.225
F-Cho (mg.min.dL^{-1})	-285 ± 472	-2711 ± 1030	-2630 ± 877	0.853
HDL-Cho (mg.min.dL^{-1})	219 ± 775	-523 ± 377	396 ± 639	0.539
TG (mg.min.dL^{-1})	-443 ± 268	73 ± 471	369 ± 259	0.175
PL (mg.min.dL^{-1})	-184 ± 480	-519 ± 888	-240 ± 472	0.393
Plasma UN(mg.min.dL^{-1})	44 ± 71	8 ± 119	-89 ± 44	0.295
NEFA (μEq.min.L^{-1})	27225 ± 4143	30894 ± 12073	42281 ± 164063	0.137
GOT (IU.min.L^{-1})	-617 ± 529	-161 ± 5296	270 ± 287	0.103
GPT (IU.min.L^{-1})	13 ± 97	-173.0 ± 154	-18.6 ± 57	0.374

[1]Values are means with SD of three ewes per treatment.

[a,b,c]Different letters indicate significant difference among the treatments (at 5% level).

L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; BHBA, β-hydroxybutyrate; T-Cho, total cholesterol; F-Cho, free cholesterol; HDL-Cho, high density lipoprotein cholesterol; TG, triglyceride; PL, phospholipid; UN, urea nitrogen; NEFA, non-esterified fatty acid; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; SD, stander deviation.

Table 3.9: Correlation coefficients among the area upper or under curve for 0-3 hours of plasma ethanol, lactate, β-hydroxybutyrate, plasma metabolites, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase[1,2].

Item	Ethanol	Lactate	BHBA	Glucose
Lactate	0.727*			
BHBA	0.374	0.374		
Glucose	-0.588	-0.254	-0.915**	
T-Cho	0.219	0.480	0.128	-0.203
F-Cho	0.208	0.722*	0.128	0.077
HDL-Cho	0.196	0.237	-0.304	0.179
Triglyceride	0.692*	0.861**	0.659	-0.541
Phospholipid	0.099	-0.013	-0.133	0.183
Plasma UN	-0.672*	-0.859**	-0.330	0.139
NEFA	-0.654	-0.347	-0.365	0.244
GOT	0.682*	0.784*	0.501	-0.397
GPT	0.023	0.405	-0.573	0.651

[1]Correlations were calculated on data sets of 9 observations.

[2] The asterisk indicate significantly different (*P < 0.05 and **P < 0.01).

AUC, area upper or under curve; L-APS, low ethanol containing apple pomace silage; H-APS, high ethanol containing apple pomace silage; BHBA, β-hydroxybutyrate; T-Cho, total cholesterol; F-Cho, free cholesterol; HDL-Cho, high density lipoprotein cholesterol; UN, urea nitrogen; NEFA, non-esterified fatty acid; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase.

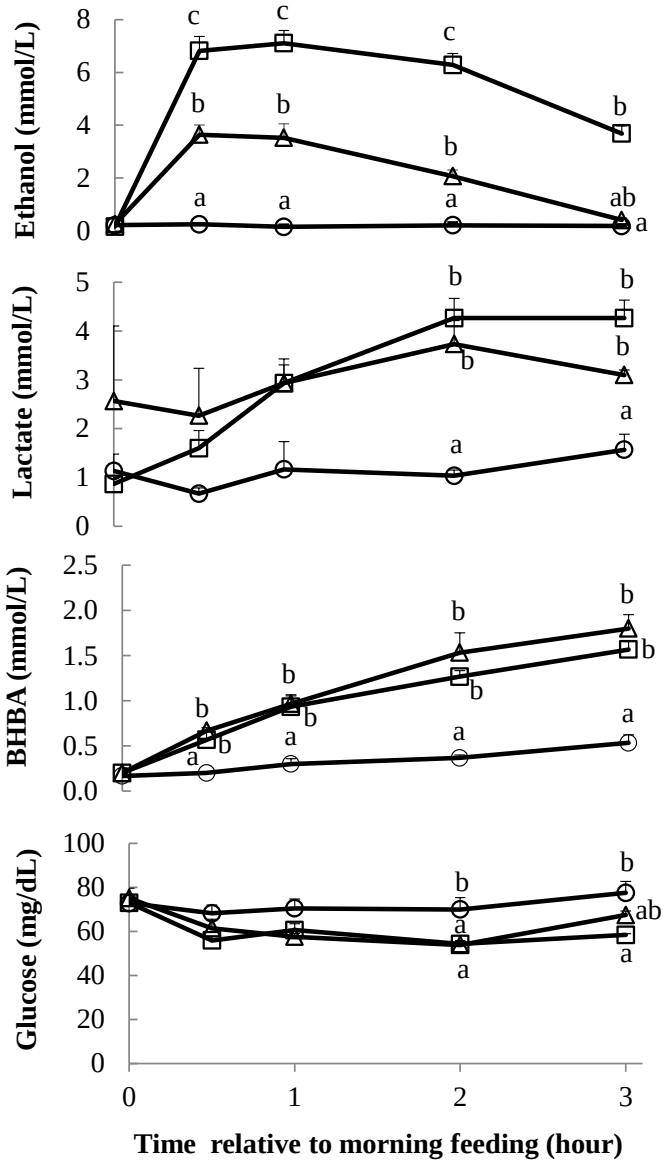


Figure 3.1: Changes in plasma ethanol, lactate, β-hydroxybutyrate (BHBA) and glucose of ewes fed on hay diet (O**)** or L-APS diet (Δ) or H-APS diet (□) for 3 hours sampling windows. The ethanol intake was calculated to be 10.61 and 15.13 g/d/head for L-APS and H-APS groups, respectively. Each data point is the mean of 3 observations ± standard error of the means. Different letters indicate significant difference among the diets (at 5% level).

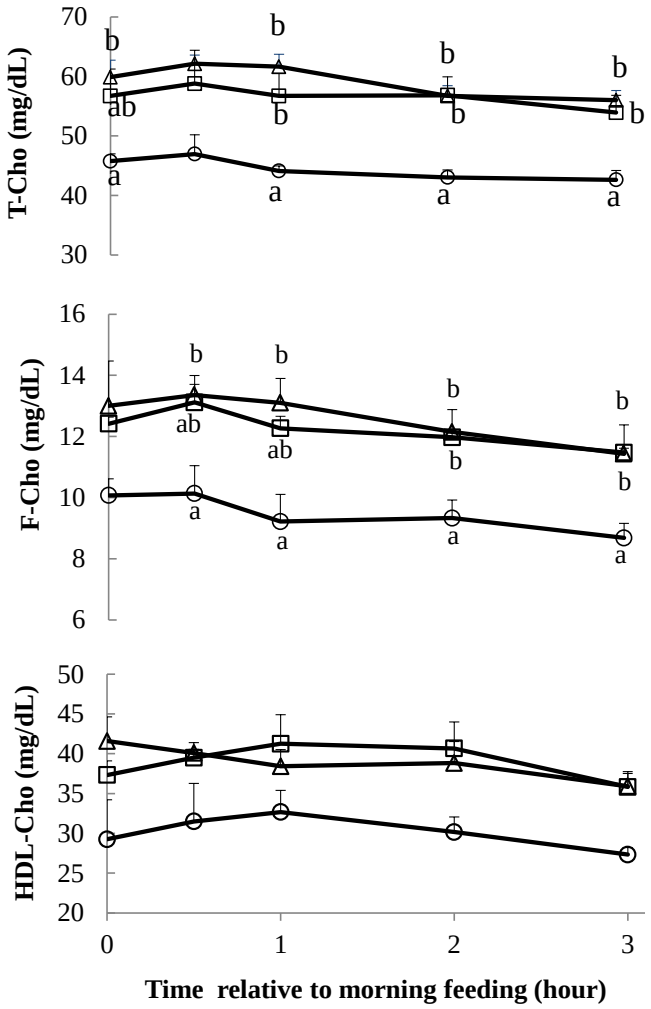


Figure 3.2: Changes in plasma T-Cholesterol (T-Cho), F-Cholesterol (F-Cho) and HDL-Cholesterol (HDL-Cho) of ewes fed on hay diet (O**)** or L-APS diet (Δ) or H-APS diet (□) for 3 hours sampling windows. Each data point is the mean of 3 observations ± standard error of the means. Different letters indicate significant difference among the diets (at 5% level).

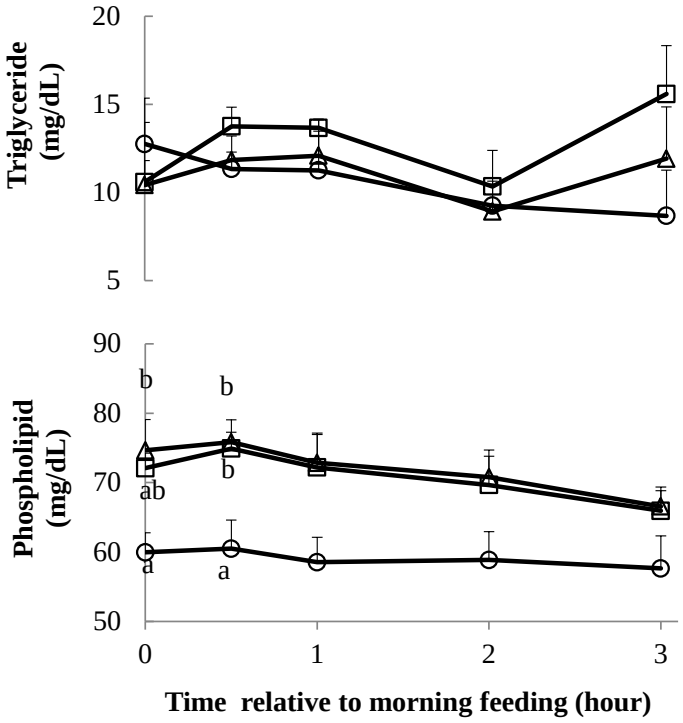


Figure 3.3: Changes in plasma triglyceride and phospholipid of ewes fed on hay diet (O**)** or L-APS diet (Δ) or H-APS diet (□) for 3 hours sampling windows. Each data point is the mean of 3 observations ± standard error of the means. Different letters indicate significant difference among the diets (at 5% level).

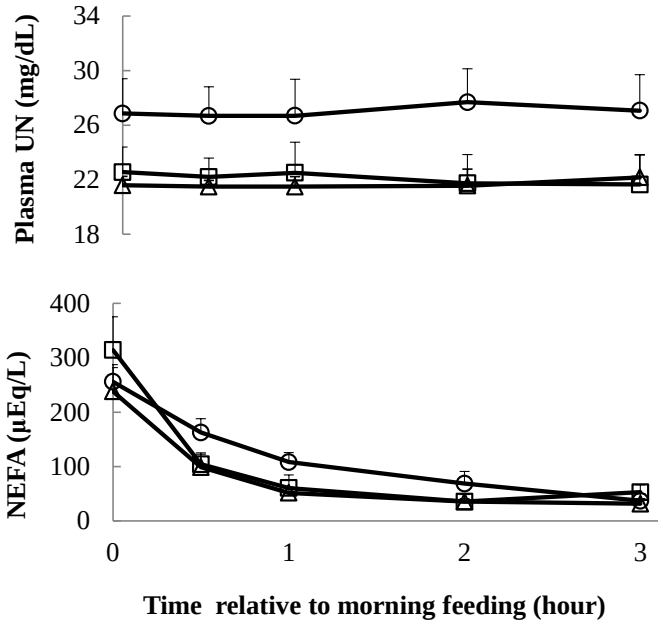


Figure 3.4: Changes in plasma urea nitrogen (UN) and non-esterified fatty acids (NEFA) of ewes fed on hay diet (O**)** or L-APS diet (Δ) or H-APS diet (□) for 3 hours sampling windows. Each data point is the mean of 3 observations ± standard error of the means.

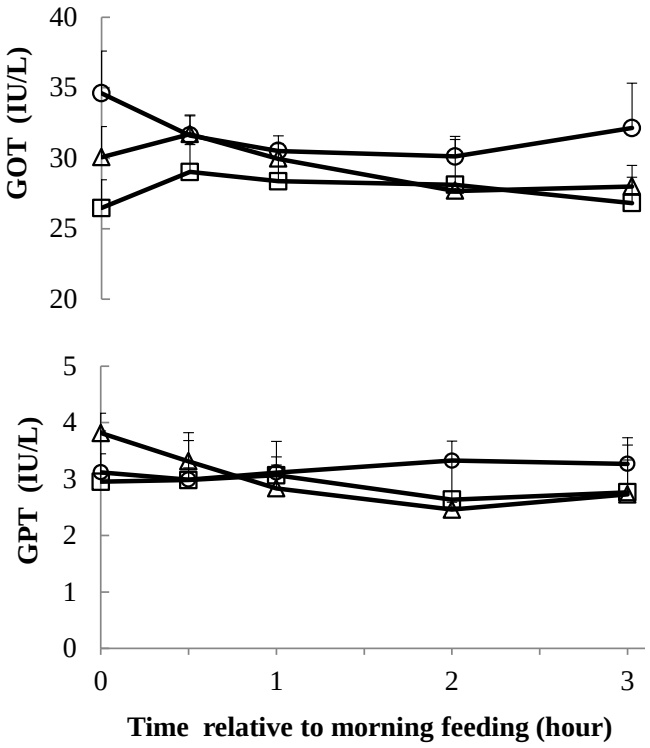


Figure 3.5: Changes in plasma glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) of ewes fed on hay diet (O**)** or L-APS diet (Δ) or H-APS diet (□) for 3 hours sampling windows. Each data point is the mean of 3 observations ± standard error of the means.

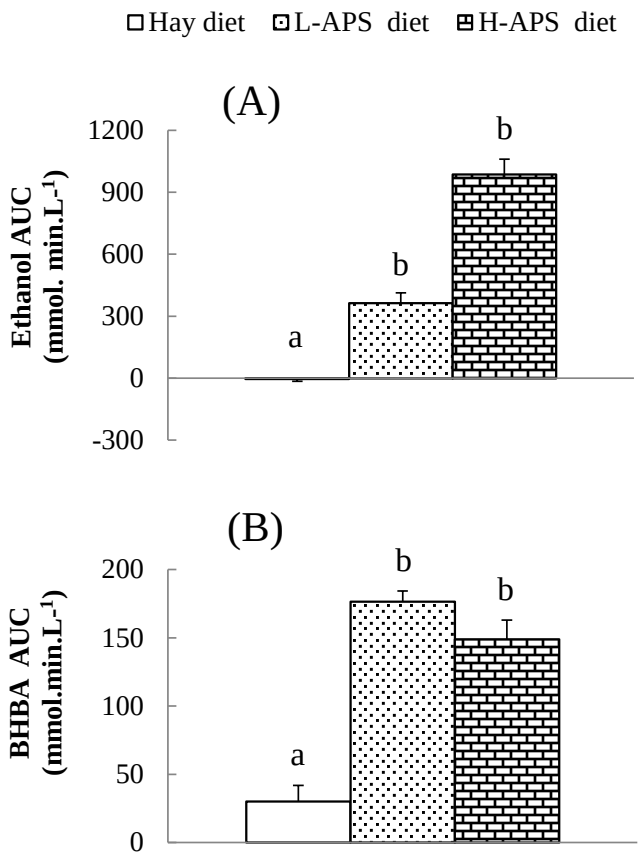


Figure 3.6: The area upper or under the curve (AUC) of plasma ethanol (A) and β-hydroxybutyrate (BHBA) (B) of ewes fed on hay diet (open bar**)** or L-APS diet (dotted bar) or H-APS diet (brick bar) for 3 hours sampling windows. Each data point is the mean of 3 observations ± standard error of the means. Different letters indicate significant difference among the diets (at 5% level).

CHAPTER 4

Effect of ethanol contained in apple pomace silage on hepatic metabolism of Suffolk sheep

INTRODUCTION

The higher amount of ethanol intake has strong effect upon hepatic gluconeogenesis. Ethanol is eliminated from the body mainly by oxidation. The liver parenchymal cell (hepatocyte) contains alcohol dehydrogenase and aldehyde dehydrogenase enzymes which oxidize ethanol into acetate. This pathway generates an overabundance of cytosolic NADH (nicotinamide adenine dinucleotide) and elevated the NADH/NAD^+ ratio (Kerbs, 1968). The consequence of the shift in the redox state is commonly held to be the mechanism responsible for inhibition of gluconeogenesis (Kerbs, 1968, Kerbs, et al., 1969). After ingestion of APS, postprandial hypoglycemia was occurred in sheep (Kondo et al., 2010, 2011). In the previous experiments (Chapter 2 and 3), it was confirmed that APS ethanol induces hypoglycemia in 2-3 hours after fed ewes and cause postprandial transient hyperketonemia through suppression of hepatic gluconeogenesis. These early studies confirmed the inhibitory effect of ethanol upon gluconeogenesis which could lead to lower hepatic glucose output. Hepatic glucose is the major determinant of plasma glucose concentration regardless of the nutritional state (Fèry, 1994). Because glycogen is the primary storage form of glucose in animal tissues and it occurs mainly in the liver, play a central role in systemic glucose homeostasis. Glycogen in muscle provides a reservoir of glucose for muscle activity, whereas liver glycogen is involved in the regulation of blood glucose levels by storage of excess glucose after meals and the subsequent release of glucose between meals. Thus dietary ethanol may have an important role for glycogen synthesis. Although surplus supply of glucose has often been shown to down regulate hepatic glucose production (Bartley and Black, 1966; Rigout et al., 2002) and a down regulation of gluconeogenic enzymes could have consequences extending beyond the

period of surplus glucose or gluconeogenic substrate supply in dairy cow (Al-Trad et al., 2010). Hesketh et al. (1998) also reported that gluconeogenesis in ruminants occurs mainly in the liver and possibly through the regulation of gene expression of key enzymes for gluconeogenesis in liver. Chronic alcohol consumption elicits a greater reduction on hepatic gluconeogenesis for female rat compare to male (Sumida et. al., 2005) and there are sex difference regarding the amount of hepatic fatty acid accumulation in the liver of human which may be caused by chronic alcohol consumption (Becker et al., 1996). Although limited research are available regarding the feeds on hepatic metabolism of ruminants (Zhang et al., 2009; Li et al., 2013; Van Harten et al., 2013), investigations on the impact of APS ethanol on hepatic gluconeogenesis and sex differences are limited. Therefore, the present study was undertaken to investigate the effect of dietary ethanol on blood parameters, hepatic glycogen content and gene expression of some regulatory enzymes of the intermediary metabolism such as pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBP1), glucose-6-phosohatase (G6Pase) and glycogen synthase (GS) with sex difference.

MATERIALS AND METHODS

1. Animals, experimental design and management

All procedures involving animals in this study were approved by the Institutional Animal Care and Use Committee at Hirosaki University (A08023).

Eight Suffolk sheep, aged 21 months on average, weighing 68.5 ± 3.7 kg, were used in this experiment to investigate the feeding effect of higher amount ethanol containing APS (109.4 g of ethanol/kg DM) on response of blood parameters, liver glycogen content and hepatic enzyme activities in ewes and wether. The experiment was performed using 2 × 2 factorial design over the 28 days periods including of initial 7 days for dietary adaptation. In this experiment, ewes and wether were allocated to two dietary treatments; alfalfa hay cube and either APS or a commercial concentrate (CP 15.5% and TDN 70%; control) at a ratio of 30:70 on TDN basis. During this time

two ewes and two wether were fed on APS diet and other two ewes and two wether were received the control diet. Daily allowance was offered as 110% TDN requirement for maintenance (Ministry of Agriculture, Forestry and Fisheries, 1996) at 10:00 and 18:00 in 2 equal meals. The required amount was adjusted weekly based on their BW. Animals were housed in individual pens with sawdust bedding in an indoor animal room and had free access to a trace mineralized salt block and water throughout the experimental period.

2. Apple pomace silage preparation

Fresh AP was obtained from an apple juice factory of the Farm village industry federation of Aomori prefectural agricultural cooperatives at Hirosaki, Japan and it was stored alone in an anaerobic condition for 2 months to secure higher ethanol contents before ensiling of APS. Because remaining sugars in fresh AP is fermented rapidly and efficiently turned into ethanol under solid-state fermentation condition (Hang et al., 1981). The APS was prepared with 70% fermented AP, 6% soybean meal, 12% wheat bran and 12% beet pulp to balance nutrient content similar to the commercial concentrate (CP 15.5% and TDN 70%). The fermented AP and all other ingredients were mixed thoroughly by a mechanical mixer and stuffed into plastic container, pressed sufficient to fill properly, topped with air-tight cover and ensiled for 10 months before use in the feeding experiment.

3. Feed samples and analyses

Apple pomace silage extraction procedure and determination procedure of pH, VBN, organic acid and ethanol were same as described in the previous part (Chapter 2.1). All feed samples were dried in a forced-air oven at 60 °C for 48 hours, ground through a 1mm screen using a Willey mill and kept in a plastic airtight container for chemical analyses. Dry matter (DM), crude protein (CP), crude fiber (CF), ether extract (EE), ash, neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by following the methods as described in the previous part (Chapter 2.1).

The pH value, organic acids and other fermented products of fermented AP and chemical composition of feed ingredients are presented in the previous experiment (Chapter 2.2). The pH value, organic acids and other fermented products in APS are presented in Table 4.1. Chemical compositions of experimental feeds are presented in Table 4.2. Fermented AP contained the largest amount of ethanol (Table 2.2.3) and thus ethanol content of APS was higher (Table 4.1). Ethanol intake was calculated from APS intake and ethanol content of the APS.

4. Blood samples collection and analytical methods

Blood samples were collected weekly immediately before morning feed, and at exactly after 3 hours of morning feed immediately before slaughtering on the last day of 28 day period by jugular venipuncture. The blood was collected into two 7 mL evacuated tubes containing sodium heparin as an anticoagulant, chilled on ice and centrifuged (3000 × g for 15 min at 4 °C) to harvest plasma sample. Plasma was aliquotted and stored at -30 °C until analyzed for ethanol, lactate, β-hydroxybutyrate (BHBA), insulin and blood metabolites [glucose, non-esterified fatty acids (NEFA), plasma urea nitrogen (UN), total cholesterol (T-Cho), free cholesterol (F-Cho), high density lipoprotein cholesterol (HDL-Cho), phospholipid and triglyceride]. All plasma samples were assayed by the following procedures as described in the previous part (Chapter 2.1).

5. Liver, kidney and Internal organs sampling

Animal of each treatment group was slaughtered in two consecutive days at the morning following on the last day of 28 day period. In the 1st slaughtering day, four ewes (two animals from each treatment) were randomly selected and slaughtered at 3 hours after the morning feed. Next day, other four wether (two animals from each treatment) were slaughtered at the same order. The animal was killed and its throat was cut. Liver and kidney samples were immediately dissected, rapidly washed by phosphate buffer saline (PBS), weighed and frozen in liquid nitrogen. Liver samples

were stored at -80 ºC until analyzed for liver glycogen content and mRNA of PC, PEPCK, FBP1, G6Pase and GS. Kidney samples were also stored at -80 ºC until analyzed for kidney glycogen content.

After that oesophagus was ligated, the animal was bled and blood weight was recorded. During the initial processing, the weights of spleen and pancreas were measured. After the rectum was ligated, the entire alimentary tract was removed and weighed. The omentum and mesenteric fat from each part was then separated and weighed. The weights of the small and large intestines (fat- and digesta-free) were obtained after removal of the digesta by hand. The weight of rumen-reticulum, omasum, and abomasum were obtained after each part was emptied. The liver and emptied gall bladder weights were also measured.

6. Tissue glycogen determination methods

Hepatic glycogen concentration was converted to glucose by the acid hydrolysis as described by Zhang (2012). About 20 mg of liver/kidney tissue was pulverized with 0.5 ml of pre-heated 2M HCL and boiled for 1 hour. To achieve complete hydrolysis, mince the liver/kidney sample within 5 minutes and shake the tube vigorously at 10 minutes during the whole process. Cool the sample at room temperature and centrifuge briefly. Neutralize the hydrolysis products with 0.5 ml of 2M NaOH solution, vortex and centrifuge (22,000 × g for 10 minutes). Supernatant part was used for glucose determination. Glucose concentration was measured as described in the previous part (Chapter 2.1). Liver/kidney glycogen content was expressed as glucose concentration (mg/g wet liver).

7. Geen expression of Gluconeogenic enzymes

7.1. RNA extraction and cDNA synthesis

Total RNA from sheep liver sample (20 mg) was lysed and homogenized with buffer RLT Plus (guanidine thiocyanate buffer, QIAGEN) and isolated using a Oiagen Mini RNeasy column (QIAGEN GmbH, Germany) following the protocols provided by

the manufacturer (QIAGEN). The concentration and purity of the RNA were determined by the NanoDrop 2000 Spectrophotometer UV-Vis (Thermo scientific, Wilmington, DE) at 260 and 280nm. For a reverse transcription (RT; RNeasy-Plus-Mini-Kit) reaction, 0.2 µg of purified RNA extracted of each sample was typically used. I. 0.2µg of RNA sample was combined with Nuclease-free-water for total volume of 6µL and incubated at 65ºC for 5 minutes using Thermal Cycler (PC 320, ASTEC) and then quickly chilled on ice. II. RNA sample was again combined with 2µl of 4×DN Master mix (g DNA Remover) and incubated at 37ºC for 5 minutes using Thermal Cycler (PC 320, ASTEC). III. RNA sample was combined with 2µl of 5×RT Master mix II (ReverTra Ace qPCR RT Master Mix, Toyobo) and incubated at 37ºC for 15 minutes and 98 ºC for 5 minutes using Thermal Cycler (PC 320, ASTEC). The Nuclease-treated RNA samples were then reverse transcribed to cDNA.

7.2. Quantification of regulatory enzymes mRNA **by** *Real-Time PCR*

Prior to designing quantitative PCR primers against the sheep nucleotide sequences, ovine cDNA clones were generated using primers designed against orthologs and are available upon request (Table 4.6). Degenerate primers were pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBP1), glucose-6-phosohatase (G6Pase), Glycogen synthesis (GS) and Beta-actin (ACTB) (Table 4.6). To avoid genomic DNA amplification, primers were designed to be located in two different exons. In order to select the most stable internal control gene under this experimental conditions, one potential housekeeping gene (ACTB) was tested. Real-time reaction were performed in a total 20 µl reaction volume containing 11.2 µl of SYBR Green PCR Master mix [Primer reverse 0.6µl (10 pmol/µl), Primer forward 0.6µl (10 pmol/µl) and SYBR Green 10µl] and 8.8 µl of sample (7 µl of cDNA Nuclease-free-water treated sample and 693 µl DEPC). ACTB is diluted for 200 fold and PC, PEPCK, FBP1, G6Pase, GS were diluted for 100 fold of the cDNA. Calibration curve was adjusted with Standard cDNA Mix created by × 4, × 16, × 64, × 256, × 1024 dilution and control cDNA Mix created by 100 µl of 8 individual cDNA

test samples. All samples were run in triplicate on a 96-well optical reaction plate. The reaction (Opticon 2, BioRad MJ) was using the following conditions: 60 seconds at 95ºC, 40 cycles (15 seconds at 95 ºC and 45 seconds at 60 ºC) and 55 ºC for 5 seconds plus 95 ºC for 5 seconds. All procedure involving gene expression is carried out at the Gene Research Center of Hirosaki University.

8. Calculation

Calculation procedure of hemicellulose, NFC, BWG, TDN intake and FCR were same as described in the chapter 2.1. Ethanol intake was calculated from APS intake and ethanol content of the APS. The relative amount of mRNA was calculated using the $^{\Delta}$Ct method using ACTB as an internal control.

9. Statistical analysis

Performance traits, liver glucose concentration, internal organs weight/body weight (%) and mRNA concentration data were analyzed using the general linear model (GLM) procedure of SPSS. Treatment, sex and treatment × sex were included in the model. Serial data for blood parameters were analyzed with polynomial contrasts to determine the slop response of the time influence and their interaction with treatment and time. Treatment means were separated using TUKEY post hoc test, protected by overall F-test. Difference was considered statistically significant at $P < 0.05$ and considered a tendency toward significance at $0.05 \leq P < 0.10$.

RESULTS

1. Performance traits

No health problems were observed in any of the ewes and wether throughout the experimental time. Mean value with standard deviation for performance traits are presented in Table 4.3. No difference was observed in initial and final BWs between the treatment groups. In like manner, there was no significant difference between the sexes in initial and final BWs. The average BWG was higher ($P < 0.01$) in the APS

group than control group in this experiment. The average BWG of wether was higher ($P < 0.01$) in the APS than control wether. The weekly cumulative live weight gain for ewes and wether during the experiment is shown in Figure 4.1. It is evident in the figure that there was gradual increase in BWG and this trend was significantly ($P < 0.05$) increased in APS receiving wether than that of control wether. Average TDN intake did not differ between treatment groups and sex. The weekly cumulative TDN intake for ewes and wether during the experiment is shown in Figure 4.2. Cumulative TDN intake was gradually increased and this trend continued throughout the experimental time for both diets; however, there was no significant difference. The FCR for APS was tended to be better ($P = 0.09$) than that of control for treatment and sex. The APS treatment group received an average of 31.4 g/d of ethanol (32.1 and 30.7 g/d for ewes and wether, respectively) whereas control group was assumed to receive any of alcohol.

2. Blood parameters

Time course changes of plasma ethanol are illustrated in Figure 4.3. After feeding of APS, weekly pre-feeding plasma concentration of ethanol was tended to increase ($P = 0.07$) and peaked at 2 weeks. These elevated concentrations for APS treatment was eventually returned to the previous values (0 week) at 4 weeks after receiving of APS (Fig 4.3A). Postprandial plasma ethanol concentration was significantly ($P < 0.01$) increased in APS receiving group at 3 hours after the morning feed; however, no significant treatment difference was detected for sex (Fig 4.3B).

Time course changes of plasma lactate are illustrated in Figure 4.4. After ingestion of APS, weekly pre-feeding plasma lactate concentrations were not influenced by treatment (Fig 4.4A). Plasma lactate concentrations for both treatments increased ($P < 0.01$) at 3 hours after the morning feed however no significant treatment and sex difference were observed (Fig 4.4B).

Time course changes of plasma BHBA are illustrated in Figure 4.5. After ingestion of APS, weekly pre-feeding plasma concentrations of BHBA were tended to

gradually declined (P = 0.06) and the lowest level was observed at 3 week (Fig 4.5A). Postprandial plasma BHBA level was significantly increased (P < 0.05) in APS treatment group at 3 hour after morning feed however no significant sex difference was detected (Fig 4.5B).

Time course changes of plasma insulin are illustrated in Figure 4.6. After ingestion of APS, postprandial plasma concentrations of insulin for both treatments were declined at 3 hour after morning feed however no significant difference was detected.

Time course changes of plasma glucose are illustrated in Figure 4.7. Weekly pre-feeding plasma glucose concentrations were tended to increased (P = 0.06) in APS receiving group than that of control (Fig 4.7A). Postprandial plasma glucose concentration for both treatment were declined after 3 hours of morning feed (P < 0.01), however, no significant treatment effect was detected although the APS treatment showed somewhat large magnitude of postprandial hypoglycemia. Postprandial plasma glucose declined level was tended to greater (P < 0.1) for APS receiving ewes (Fig 4.7B) than that of control ewes.

Time course changes of plasma T-Cho, F-Cho, HDL-Cho and phospholipid are illustrated in Figure 4.8, 4.9 4.10, and 4.11, respectively. After ingestion of APS, weekly pre-feeding plasma concentrations of T-Cho, F-Cho, HDL-Cho and phospholipid were significantly increased (P < 0.01, P < 0.05, P < 0.05 and P < 0.05, respectively) and the highest level was detected at 4th week (Figure 4.8A, 4.9A, 4.10A and 4.11A, respectively). Weekly pre-feeding plasma concentration of T-Cho was tended (P = 0.09) to influence by sex however F-Cho, HDL-Cho and phospholipid concentrations were not influenced by sex. Postprandial plasma T-Cho, F-Cho, HDL-Cho and phospholipid concentrations for APS treatment were increased (P < 0.05, P < 0.05, P < 0.05 and P < 0.01, respectively) after 3 hours of morning feed, however postprandial plasma concentrations were not influenced by sex (Figure 4.8B, 4.9B, 4.10B and 4.11B, respectively).

Time course changes of plasma triglyceride are illustrated in Figure 4.12. After ingestion of APS, weekly pre-feeding plasma concentrations of triglyceride were not influenced by treatment and sex (Fig 4.12A). Postprandial plasma triglyceride concentration for APS treatment was increased ($P < 0.05$) after 3 hours of morning feed however postprandial plasma tri-glyceride concentration was not affected by sex (Figure 4.12B).

Time course changes of plasma NEFA are illustrated in Figure 4.13. After receiving of APS, pre-feeding plasma NEFA concentration was not affected by treatment however plasma NEFA concentration tended ($P = 0.09$) to influence by sex (Figure 4.13A). Postprandial plasma NEFA concentration was not affected by treatment however plasma NEFA concentration affected ($P < 0.01$) by sex (Figure 4.13B).

Time course changes of plasma UN are illustrated in Figure 4.14. After feeding of APS, plasma UN concentration was not affected by treatment and sex for weekly pre-feeding and post-feeding levels also (Figure 4.14A and 4.14B, respectively).

3. Liver tissue compositions

Liver glucose concentration was lower in APS receiving group however no significant difference was detected between the APS and control treatments (Figure 4.15). Liver glucose concentration was not influenced by sex.

No significant difference was detected between the APS and control treatments for the liver triglyceride, T-Cho, phospholipid and NEFA concentrations (Table 4.4).

4. Kidney glycogen content

No glucose molecule was detected in the kidney tissue.

5. Internal organ weight per BW (%)

The effects of APS feeding on internal organs weight per BW (%) of sheep are presented in Table 4.5. After 4 weeks of APS ingestion, the liver weight per BW (%) was markedly affected ($P < 0.01$) by treatment and APS receiving group had heavier liver than that of control treatment. However, there was no sex difference for the liver

weight per BW (%). After feeding of APS diets, the internal organ, total blood, pancreases, spleen, gallbladder, omasum, abomasum, small intestine and large intestine weights per BW were not influenced by treatment and sex. The rumen-reticulum, omentum and mesenteric fat weights per BW (%) were not affected by treatments however sex difference ($P < 0.05$, $P < 0.01$ and $P < 0.1$, respectively) was existed between APS and control feed receiving animals.

6. Quantification of regulatory enzymes mRNA by Real-Time PCR

Quantification of mRNA expression by real-time PCR in gluconeogenic pathways is presented in Table 4.7. The PC expression in the alcoholic sheep liver was 1.4-fold greater, however, no significant difference was detected between the APS and control groups (Figure 5.16). After feeding, the mRNA expression of PEPCK was tended ($P = 0.06$) to higher (2.6-fold greater) in APS ethanol receiving sheep liver compared with control (Figure 5.16). The expression of FBP1 for the APS group was 1.2-fold greater however no significant differences were observed (Figure 5.16). G6Pase expression in the alcoholic sheep liver was 4.3-fold greater ($P < 0.05$) than that of control sheep liver (Figure 5.16). The expression of glycogen synthesis (GS) was not significantly differed between the APS and control groups (Figure 5.16). There was no sex difference between the APS and control diets for genes expression.

DISCUSSION

In the present study, highest ethanol content was observed for fermented APS (109.4 g/kg DM) in comparison with other fermented APSs in (chapter 2 and 3) that blended with same ingredients to improve nutritional balance of AP after 2 month storage period under similar anaerobic condition. The APS contained about 5-6 times greater ethanol content compared to corn, grass and TMR silages (McDonald et al., 1991; Raun and Kristensen, 2010; Lawrence et al., 2011; Cummins et al., 2009). Hepatic gluconeogenesis is a key component of total glucose entry in cattle (Huntington et al., 2006). Consequently, the regulation of gluconeogenesis is an area

of great interest and the effects of APS ethanol ingestion upon the liver and impact of sex has been expended by this study.

After 4 weeks of APS ingestion, the average BWG was significantly increased in the APS than control treatment in this experiment. The average BWG was 30% higher for ewes of APS group. A tendency for increased FCR was also observed in the APS than control treatment. Taasoli and Kafilzadeh, (2008) also reported the improved ADG and FCR by feeding of both ensiled and dried AP in finishing lambs. We have formulated daily ration to meet 110% TDN requirement for maintenance (Ministry of Agriculture, Forestry and Fisheries, 1996; Japan) where APS or concentrate provided 70% TDN requirement and we found 83, 110 and 57 g/d BWG for APS group, ewes and wether, respectively. In this study, sufficient energy was provided from APS and sheep grew faster than the targeted daily maintenance level. One week before starting the experiment, wether were newly arrived from Kanagi farm, Hirosaki University experimental station, naturally the animal were in stress which resulted in negative or less body weight gain compare to ewes. Therefore, ingested dose of ethanol (~31.4 g/d) from APS in this study would not cause any harmful influence on the performance of growing ewe and wether.

After 4 weeks of APS ingestion, pre-feeding plasma ethanol concentrations was 1.5 fold higher in APS sheep compared to control at 2nd week. After ingestion of APS, postprandial plasma concentration was rapidly increased and APS group had 69 fold higher plasma ethanol level than that of control at 3 hours after feeding and 22 fold higher within group from pre-feeding to post-feeding time related to morning feed. Postprandial plasma BHBA concentration was also rapidly elevated for APS group at 3 hour after morning feed. Kristensen et al. (2007) also observed increases in plasma concentrations of ethanol and BHBA after feeding of corn silages which contained a measurable amount of ethanol (14.2 g/kg DM), however they concluded that typical amounts of alcohols in corn silage do not interfere with splanchnic metabolism of major metabolites and do not saturate hepatic pathways for alcohol metabolism. In our observation, APS contained about 8 times greater ethanol content compared to corn

silage which was reported by Kristensen et al. (2007). Previous chapter 2 and 3 confirm the inhibitory effect of APS ethanol upon gluconeogenesis resulting lower glucose concentration, which could lead to ethanol-induce hyperketonemia.

According to the results of kinetic study of infused ethanol in sheep, Jean-Blain et al. (1992) assumed that daily ethanol intake ranging from 0.2 to 1 g/kg BW can be metabolized by rumen microflora and enzymatic system of the host and plasma ethanol level remains below 0.25 g/L. In the fact the sheep consumed 0.45 g/kg BW of ethanol and the highest plasma ethanol level was 0.35 g/L observed in the present study which exited the recommended limit. Excess amounts of ethanol may influence in higher oxidation of ethanol into acetate and rapidly inhibit gluconeogenesis which leads to greater production of BHBA. Veenhuizen, et al. (1991) also reported that hepatic in vitro gluconeogenic capacity decreased significantly for ketosis cow and at kitotic stage plasma BHBA increased 3.5~8.4 fold by using feed restriction plus dietary 1,3-butanediol. In the fact the sheep consumed APSs ethanol and plasma BHBA increased dramatically 5.8 fold for peaked hour which indicated that ketosis occurred in this experiment also. It is established that ethanol inhibit hepatic gluconeogenesis from lactate, glycerol, some amino acids and other substrates (Krebs et al., 1969) and postprandial hypoglycemia in sheep due to ingestion of APS diets (Kondo et al., 2010; 2011). In support, the previous experiments (chapter 3) also found that postprandial plasma glucose concentration was declined due to APS ethanol ingestion in ewes. After APS ingestion, plasma lactate was elevated for wether compare to ewes. It was unexpected that after ingestion of APS, pre-feeding plasma glucose levels were tended to increase in APS treatment and pre-feeding plasma glucose level was higher for wether compare to ewes. Postprandial hypoglycemia was occurred for ewes in APS receiving group than that of control ewes after 3 hours morning feed. Thus, the lower concentration of lactate uptake by ewes resulted in lower rate of gluconeogenesis. It would be reported that some of the decline in plasma glucose concentration is due to a distinct reduction in hepatic gluconeogenesis capacity in ethanol ingested Suffolk ewes.

Similarly, Sumida et al. (2005) also reported a greater alcohol induced suppression of hepatic gluconeogenic in female rats fed the ethanol diet.

After ingestion of APS, pre-feeding and post-feeding plasma T-Cho, F-Cho, HDL-Cho and phospholipid were increased. After feeding, postprandial plasma triglyceride level was also elevated in this experiment. This indicated that after APS ethanol feeding, sheep had high levels of total cholesterol, phospholipid and triglyceride as well as increased liver triglyceride in comparison with control treatment. The negative effects of NADH: NAD^+ on hepatic gluconeogenesis, fatty acid oxidation is reduced. The elevated NADH/NAD^+ ratio inhibit the gluconeogenesis (Kerbs, 1968) and thus cause the higher amount of BHBA and accumulated fat in the liver. Holtenius and Holtenius (1996) reported that primary factor for the ketotic state is the high demand for glucose and insufficient gluconeogenesis, which is compensated by increased ketogenesis, there are very few prerequisites for lipid synthesis in the liver cell and small risks for fat accumulation in ruminants. Accumulation of fat in the liver can be elevated by secreting lipids into the blood steam. Accompanying with the combination of higher dietary fat and ethanol has a synergistic effect in tended to increase plasma lipid concentration because ethanol suppresses the clearance of intestinally derived chylomicrons. Alcoholic beverage consumption is associated of greater HDL-Cho in humans which may cause by ethanol reported by others (Henk, et al., 1998; Frohlich, 1996; Minna, et al., 2003; Van der Gaag et al, 2001). However, weekly pre-feeding T-Cho level was tended to influence by sex but we have failed to detect any significant sex difference in others lipid profiles. After 4 week feeding trial, APS receiving group showed 20% heavy liver than that of control treatment. The elevated fat composition of the APS receiving group could be a factor for the increase in liver weight. Sumida et al. (2005) suggested that an interaction between the high fat content of the diet and ethanol, which could contribute to the elevation in liver weight. In support, APS contained higher amount of dietary fat compare to control. After 4 weeks ingestion, the APS ethanol receiving group revealed a decrease of 27% in liver glucose levels as well as liver glycogen content had depleted than that of control group however no significant

treatment difference was detected. Barid et al. (1968) and Ballard et al. (1968) demonstrated that the hepatic glycogen concentration was lower in the ketotic cow than in the healthy lactating cow which is correspond very well to our observations. Well-fed animals like growing sheep in the present study would have sufficient precursors for ketogenesis. Therefore once ingested ethanol suppressed gluconeogenesis, lactate would become redundant in gluconeogenic pathway and be accumulated and BHBA would also be generated from fatty acids in well-fed ruminant animals. Consistent heavy liver for APS receiving group in this study are strongly supporting this notion.

PEPCK and G6Pase are two key enzymes that drive gluconeogenesis and PEPCK is the rate-limiting enzymes (Rognstad, 1979; Donkin, 1999) whereas G6Pase catalyzes the final step of gluconeogenesis, the production of free glucose from glucose 6-phosphate (Xing-Hai, 2006). Lower gluconeogenesis at the PEPCK branch play a secondary role in supplying carbon for glucose production, the latter being driven predominantly by G6Pase activity. After ingestion of alcoholic APS, the mRNA expression of both key enzymes (PEPCK and G6Pase) were tended to increase or increased (2.5 and 4.2-fold greater, respectively) in APS receiving sheep liver. This study is the first to report that mRNA expression increases for rate-limiting gluconeogenic enzymes of PEPCK and G6Pase by feeding alcoholic fermented APS through suppression of hepatic gluconeogenesis. Li et al. (2013) also observed higher or tended to be higher of PEPCK and G6Pase mRNA expression for corn silages receiving group, however they were not considered of silage ethanol whereas another group received without corn silage diet and they concluded that different dietary ingredients could change gene expression of hepatic gluconeogenesis pathway. Pilkis and Granner (1992) also reported hepatic PEPCK activity is markedly increased in fasted or diabetic animals. Consistent plasma glucose declined at 2-3 hours after morning feed for APS group in the previous experiments (Chapter 3) and postprandial hypoglycemia at 3 hour after morning feed for APS receiving ewes of this experiment are strongly supporting this notion. Kondo et al. (2010; 2011) also reported the postprandial lower plasma glucose concentration due to ingestion of APS ethanol.

Fructose 1,6-bisphosphatase is the enzyme that releases fructose 6-phosphate from the gluconeogenic pathway (Pilkis and Granner, 1992), which after conversion to glucose 6-phosphate, can release glucose by the action of G6Pase. Fructose 1, 6-bisphosphatase thus control the overall output of gluconeogenesis regardless of the precursors utilized by the action of FBP1. Although, we have failed to detect the significant increase of FBP1 for APS receiving sheep liver. Thorn, et al. (2012) also reported that hepatic gluconeogenic genes (PEPCK, G6Pase, FBP1, GLU2 and PGCIA) expression increased in the hypoglycemic sheep fetuses induced by maternal insulin infusion for 8 weeks which are correspond very well to our observations. PC is the first regulatory enzyme in gluconeogenesis pathway that converts pyruvate to oxaloacctate in gluconeogenesis (Pershing et al., 2002). Although we failed to demonstrate significant changes of PC between two groups however PC expression was 1.4-fold greater in the alcoholic sheep liver. GS, the key rate-limiting enzyme associated with glycogen synthesis was not influence by the APS ethanol which indicates that APS ethanol does not affect hepatic glycogen content. Rozance et al. (2008) also reported that hepatic PEPCK and G6Pase mRNA increased 12-fold and 7-fold, respectively, following chronic hypoglycaemia with no change in hepatic glycogen. This statement is correspond very well to this present study, however our findings were somewhat smaller. Therefore, our study demonstrated that in order to respond of APS ethanol, genes involved in gluconeogenesis pathway played an important role and fermented APS feeding have a positive influence on the activity of key gluconeogenesis enzymes in alcoholic sheep. We speculated that PEPCK and G6Pase might be the most vital regulatory genes in hepatic gluconeogenesis of sheep liver for ethanol metabolism. Although all of the response observed in the blood parameters and 2 rate controlling hepatic gluconeogenic enzymes, however, liver glycogen and lipids concentration were not influenced by the APS treatment and no health problem were detected.

Based on above findings, the consistent increase in plasma ethanol, BHBA and cholesterol after feeding of alcoholic-fermented APS, heavy liver for APS receiving group and the higher mRNA expression of PEPCK and G6Pase for hepatic

gluconeogenesis we concluded that APS ethanol consumption induce postprandial transient hyperketonemia through suppression of hepatic gluconeogenesis and APS ethanol could change gene expression of hepatic gluconeogenesis pathway, and PEPCK and G6Pase might be the most vital regulatory genes in hepatic gluconeogenesis of sheep liver for ethanol metabolism. Ewes are more vulnerable to ethanol induced post prandial hypoglycemia.

SUMMARY

Eight Suffolk sheep were used to investigate the feeding effect of high ethanol APS (109.4 g of ethanol/kg DM) on response of blood parameters, liver glycogen content and hepatic enzyme activities in ewes and wether. The experiment was performed using 2 × 2 factorial design over the 28 days periods including of initial 7 days for dietary adaptation. In this experiment, ewes and wether were allocated to two dietary treatments; alfalfa hay cube and either APS or a commercial concentrate (CP 15.5% and TDN 70%; control) at a ratio of 30:70 on 110% TDN requirement for maintenance. At the beginning of each week, series of blood samples were obtained by jugular venipuncture at pre-feeding (0) and on the last morning of the feeding trial (28th day) blood samples were obtained by jugular venipuncture at pre-feeding (0) and post-feeding (3 hours after the feeding and immediately before slaughtered the animals). All plasma samples were assyed for ethanol, lactate, β-hydroxybutyrate (BHBA), insulin, glucose, non-esterified fatty acids (NEFA), plasma urea nitrogen (UN), total cholesterol (T-Cho), free cholesterol (F-Cho), high density lipoprotein cholesterol (HDL-Cho), phospholipid, and triglyceride. The animal was killed and its throat was cut. Liver samples were immediately dissected, rapidly washed by phosphate buffer saline (PBS), weighed and frozen in liquid nitrogen. Liver samples were stored at -80 ^{o}C until analyzed for liver glycogen content and gene expression of pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBP1), glucose-6-phosohatase (G6Pase), glycogen synthase (GS). After slaughtered, blood, total internal organs, liver, pancreases, spleen, gallbladder,

rumen-reticulum, omasum, abomasum, small intestine, large intestine, omentum and mesenteric fat weight were recorded. After ingestion of APS, the pre-feeding and post feeding plasma ethanol, T-Cho, F-Cho, HDL-Cho, phospholipid concentrations were increased. Weekly pre-feeding plasma BHBA concentration was tended to decrease and glucose concentration was tended to increase in APS receiving groups than that of control. Postprandial plasma BHBA and triglyceride concentrations were also increased in APS receiving group. Postprandial hypoglycaemic tendency was occurred in ewes fed on APS diet. Pre-feeding lactate concentration was higher in APS receiving wether, however there was no significant treatment effect was detected. Liver weight was significantly higher in APS receiving group. Liver glucose concentration was lower in APS group however no effect was detected. After feeding, the mRNA expression of PEPCK was tended ($P = 0.06$) to greater (2.6-fold) and G6Pase was greater ($P < 0.05$; 4.3-fold) in APS ethanol receiving sheep liver compared with control. PC, FBP1 and GS expressions were not influenced by the APS ethanol in treatment and sex.

Based on above findings, the consistent increase in plasma ethanol, BHBA and lipid profiles after feeding of alcoholic-fermented APS, heavy liver for APS receiving group and the higher mRNA expression of PEPCK and G6Pase for alcoholic liver group, we concluded that APS ethanol consumption induce postprandial transient hyperketonemia through suppression of hepatic gluconeogenesis, there have a little risks for fat accumulation in the liver and APS ethanol could change gene expression of hepatic gluconeogenesis pathway where PEPCK and G6Pase might be the most vital regulatory genes in hepatic gluconeogenesis of sheep liver for ethanol metabolism. Ewes are more vulnerable to ethanol induced postprandial hypoglycemia.

Table 4.1: The pH values, organic acids and others fermented products of apple pomace silage.

Items	**APS**
pH	3.7
Organic acids and others fermented products (g kg $^{-1}$ DM)	
Lactic acid	56.4
Acetic acid	19.9
Propionic acid	0.4
Normal butyric acid	ND
Iso-butyric acid	ND
Ethanol	109.4
VBN in TN	0.7
Moisture	666.7

APS, apple pomace silage; g, gram; kg, kilogram; DM, dry matter; ND, not detectable; VBN, volatile basic nitrogen; TN, total nitrogen.

Table 4.2: Chemical compositions of experimental feeds.

Items	**Alfalfa hay cube**	**Concentrate**	**APS**
Dry matter (%)	89.7 ± 0.6	88.9 ± 0.6	32.6 ± 0.7
***Nutrient composition* (*%, DM basis*)**			
Organic matter	87.9 ± 0.5	93.1 ± 0.1	93.4 ± 0.1
Crude protein	18.1 ± 0.0	17.3 ± 0.2	19.4 ± 0.2
Ether extract	2.2 ± 0.0	3.1 ± 0.1	4.0 ± 0.1
Crude ash	12.1 ± 0.4	6.9 ± 0.1	6.6 ± 0.1
ADF	30.1 ± 0.8	3.8 ± 0.2	19.5 ± 0.9
NDF	43.5 ± 0.5	19.5 ± 0.6	34.6 ± 0.3
Hemicellulose	13.4 ± 0.2	15.8 ± 0.4	15.1 ± 1.1
NFC[1]	31.0 ± 0.1	57.7 ± 1.0	45.7 ± 0.1

[1]NFC = (100 - CP - EE -NDF - Ash).

APS, apple pomace silage; DM, dry matter; NFE, nitrogen free extract; ADF, acid detergent fibre; NDF, neutral detergent fibre; NFC, non-fibrous carbohydrate.

Table 4.3: Body weights and performance traits in the sheep[1].

Items	Control treatment			APS treatment			P-value		
	Control	Ewe	Wether	APS	Ewe	Wether	T	S	T × S
Initial BW, kg	68.6 ± 4.0	70.1 ±0.4	67.2 ± 6.3	68.3 ± 4.1	70.3 ± 4.1	66.3 ± 4.1	0.910	0.322	0.855
Final BW, kg	69.4 ± 4.4	71.7 ± 0.1	67.1 ± 6.2	70.0 ± 4.5	72.6 ± 4.4	67.5 ± 4.1	0.845	0.19	0.929
Average BWG, g/d	37 ± 48.7	76 ± 23.6	-5 ± 3.4	83 ± 31.8	110 ± 16.8	57 ± 3.4	0.01	0.003	0.241
TDNI, g/d	703 ± 30.7	714 ± 2.7	692 ± 48.3	700 ± 31.3	715 ± 31.1	685 ± 32.1	0.91	0.32	0.859
FCR (TDNI/BWG)	-89.5 ± 135.1	9.8 ± 3.1	-188.9 ± 123.4	9.3 ± 3.2	6.6 ± 0.7	12.0 ± 1.3	0.086	0.091	0.08
Ethanol intake[2], g/d	0.0	0.0	0.0	31.4 ± 1.4	32.1 ± 1.4	30.7 ± 1.4	0.000	0.384	0.384

[1]Values are means with SD of four sheep per treatment (ewes/wether = 2).

APS, apple pomace silage; T, treatment; S, sex; BW, body weight; BWG, body weight gain; TDNI, total digestible nutrient intake; FCR, feed conversion ratio; kg, kilogram; g/d, gram per day.

Table 4.4: Liver lipids composition of sheep[1].

Items	Control treatment			APS treatment			P-value		
	Control	Ewe	Wether	APS	Ewe	Wether	T	S	T × S
Triglyceride (mg/g wet liver)	17.0 ± 4.5	14.0 ± 4.5	20.0 ± 2.1	19.2 ± 9.1	22.5 ± 13.3	15.9 ± 5.0	0.704	0.957	0.301
T-Cho (mg/g wet liver)	9.8 ± 2.5	9.9 ± 4.3	9.7 ± 0.4	9.5 ± 2.0	9.7 ± 3.3	9.2 ± 1.3	0.867	0.873	0.949
Phospholipid (mg/g wet liver)	79.7 ± 18.1	82.8 ± 29.9	76.7 ± 7.4	70.0 ± 8.3	67.8 ± 13.1	72.2 ± 4.1	0.461	0.947	0.681
NEFA (μEq/g wet liver)	13.1 ± 4.3	15.5 ± 5.7	10.7 ± 0.2	12.0 ±2.5	13.4 ± 3.3	10.6 ± 0.7	0.662	0.181	0.682

[1] Values are means with SD of four sheep per treatment (ewes/wether = 2).

APS, apple pomace silage; T, treatment; S, sex; mg, miligram; g, gram; SD, standard deviation.

Table 4.5: Effect of APS feeding on blood and internal organs weight per body weight (%) of sheep[1].

Items	Control treatment			APS treatment			P-value		
	Control	Ewe	Wether	APS	Ewe	Wether	T	S	T × S
Final BW(kg)	69.4 ± 4.4	71.7 ± 0.1	67.1 ± 6.2	70.0 ± 4.5	72.6 ± 4.4	67.5 ± 4.1	0.85	0.19	0.93
Blood wt/BW, %	3.0 ± 0.6	2.7 ± 0.0	3.4 ± 0.7	3.4 ± 0.6	3.1 ± 0.2	3.8 ± 0.8	1.00	0.23	1.00
%, organs weight per BW									
Whole internal organ	21.5 ± 0.6	21.6 ± 0.9	21.5 ± 0.5	21.4 ± 0.8	21.1±1.4	21.6 ± 0.0	0.79	0.78	0.62
Liver	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	0.008	0.13	0.19
Pancreas	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.11	0.28	0.66
Spleen	0.5 ± 0.3	0.7 ± 0.3	0.4 ± 0.3	0.6 ± 0.2	0.7 ± 0.1	0.4 ± 0.1	0.88	0.14	0.78
Gallbladder	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.82	0.82	0.82
Rumen-reticulum	1.5 ± 0.2	1.4 ± 0.1	1.7 ± 0.2	1.5 ± 0.2	1.3 ± 0.0	1.7 ± 0.0	0.71	0.02	0.44
Omasum	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.17	0.16	0.30
Abomasum	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.25	0.53	0.96
Small intestine	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.55	0.12	0.88
Large intestine	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.41	0.69	0.50
Omentum	3.3 ± 1.5	4.6 ± 0.2	1.9 ± 0.1	3.0 ± 1.3	4.0 ± 0.2	1.9 ± 0.4	0.20	0.00	0.20
Mesenteric fat	1.3 ± 0.5	1.7 ± 0.4	1.0 ± 0.2	1.2 ± 0.2	1.3 ± 0.1	1.1 ± 0.2	0.44	0.08	0.24

[1] Values are means with SD of four sheep per treatment (ewes/wether = 2).

APS, apple pomace silage; T, treatment; S, sex; BW, body weight; kg, kilogram; %, percent; SD, standard deviation.

Table 4.6: Primer sets used for real-time quantitative PCR analysis.

Gene[1]	Primer[2]	Sequence[3]	Amplicon (bp)	Accession number[4]
PC	Forward	GCACAGCATGGGGCTTGGCT	126	XM_004019920.1
	Reverse	AACTGGGCCAGGTCCCCCAC		
PEPCK	Forward	TGTCCGAGGAGGATTTTGAG	124	XM_004014441.1
	Reverse	ATGCCAATCTTGGACAGAGG		
FBP1	Forward	AACCGGGCTCCAGCATGACG	92	XM_004004092.1
	Reverse	ACGGGCCTTCCTGCCCTCTT		
G6Pase	Forward	CTGGATCGTGCAACTGAATG	137	XM_004012951.1
	Reverse	TGGCATTGTAGATGCTCTGG		
GS	Forward	TTTTCAACTCAGCGGCAGTC	87	XM_004006794.1
	Reverse	TCTAATGGTGCTGAGGATGG		
ACTB	Forward	CCAGCACGATGAAGATCAAG	148	DQ386889.1
	Reverse	TAGAAGCATTTGCGGTGGAC		

[1]PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; FBP1, fructose 1,6-bisphosphatase; G6P, glucose-6-phosohatase; GS, Glycogen synthesis, ACTB, ß-actin.

[2]F, forward; R, reverse.

[3]All primer were sequenced with 5' to 3'. The 3' notation indicates the starting point for the reverse primer in the single-standard sequence used to design primers.

[4]Gene Bank or Reference Sequence.

Table 4.7: Quantification of mRNA expression by real-time PCR in gluconeogenic pathways of sheep[12].

Items	Control treatment			APS treatment			P-value		
	Control	Ewe	Wether	APS	Ewe	Wether	T	S	T × S
PC/ACTB	1.0 ± 0.5	1.2 ± 0.6	0.7 ± 0.3	1.4 ± 0.5	1.3 ± 0.5	1.5 ± 0.8	0.37	0.739	0.413
PEPCK/ACTB	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3	1.3 ± 0.4	1.4 ± 0.6	1.2 ± 0.3	0.058	0.695	0.736
FBP1/ACTB	1.3 ± 0.3	1.2 ± 0.0	1.5 ± 0.3	1.5 ± 0.4	1.4 ± 0.5	1.7 ± 0.3	0.458	0.22	0.953
G6Pase/ACTB	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	2.6 ± 1.0	2.8 ± 1.5	2.4 ± 0.7	0.032	0.767	0.696
GS/ACTB	1.1 ± 0.2	1.0 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.0	1.3 ± 0.1	0.347	0.15	0.65

[1] Values are means with SD of four sheep per treatment (ewes/wether = 2).

[2]mRNA expression was measured using real time PCR with ACTB mRNA expression as an internal control from triplicate measurements.

APS, apple pomace silage; T, treatment; S, sex;

PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; FBP1, fructose 1,6-bisphosphatase; G6Pase, glucose-6-phosohatase; GS, Glycogen synthesis, ACTB, ß-actin.

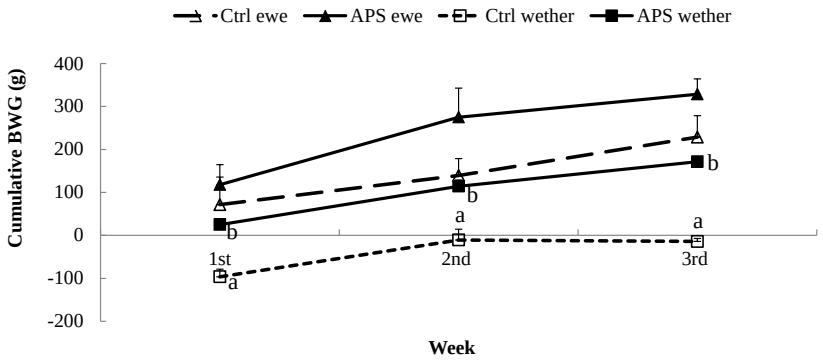


Figure 4.1: Cumulative body weight gain (BWG; g) of ewes and wether fed on control or APS diet for 21 days. Each data point is the mean of 2 observations ± standard error of the means. Different letters indicate significant difference between the sex (at 5% level).

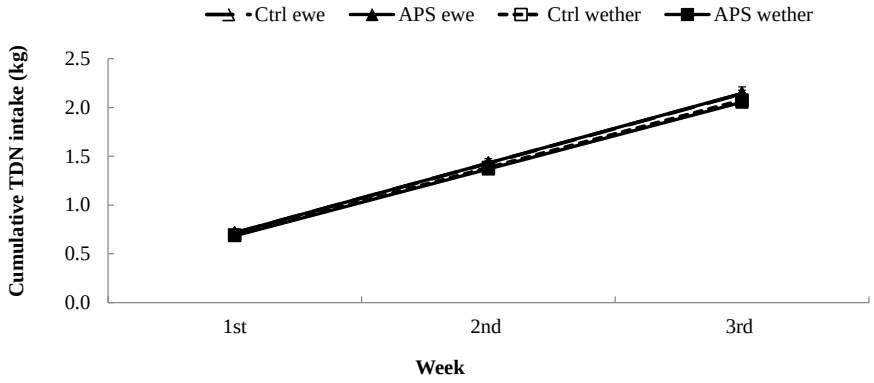
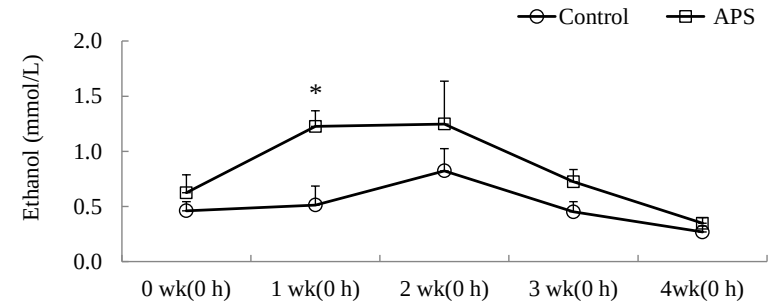
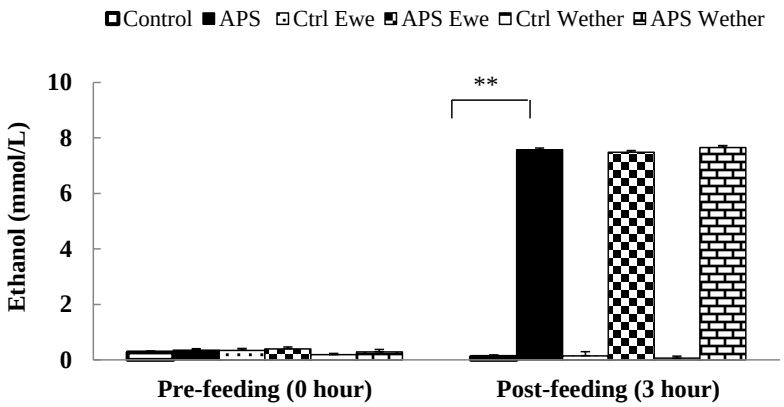


Figure 4.2: Cumulative TDN intakes (TDN intake; kg) of ewes and wether fed on control or APS diet for 21 days. Each data point is the mean of 2 observations ± standard error of the means.



(A) Weekly plasma concentration pattern relative to morning pre-feeding



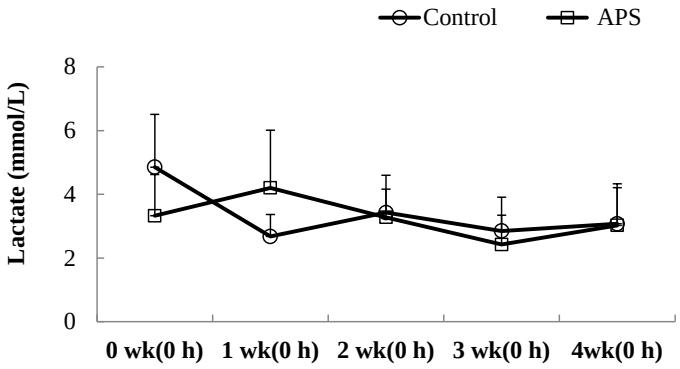
(B) Time relative to morning feeding (hour)

Figure 4.3: Time course changes of plasma Ethanol of Suffolk sheep fed on control or APS tretment. The ethanol intake in APS treatment was calculated to be 31.4, 32.1 and 30.7 g/d/head for APS treatment, ewe and wether, respectively.

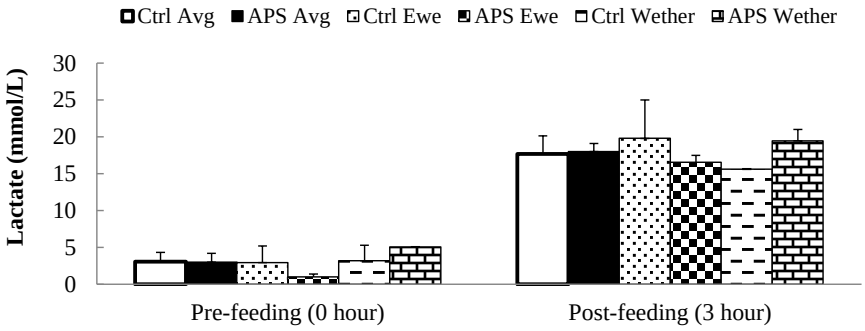
In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2). The asterisk(s) indicate the significant difference (s) between treatments at each time point (*P < 0.05 and **P < 0.01).



(A) Weekly plasma concentration pattern relative to morning pre-feeding



(B) Time relative to morning feeding (hour)

Figure 4.4: Time course changes of plasma Lactate of Suffolk sheep fed on control or APS tretment.

In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2).

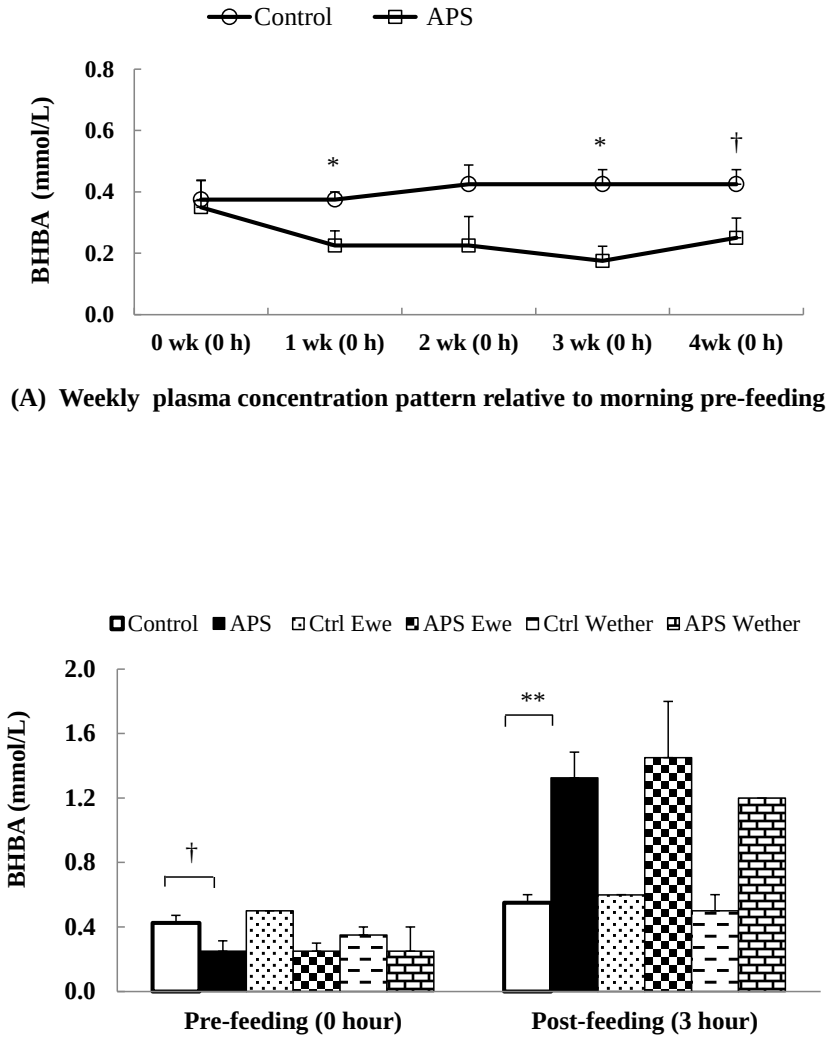


Figure 4.5: Time course changes of plasma β-hydroxybutyrate (BHBA) of Suffolk sheep fed on control or APS tretment.

In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2). The dagger or asterisk(s) indicate a tendency toward significant difference or the significant difference (s) between treatments at each time point (†$P < 0.1$, *$P < 0.05$ and **$P < 0.01$).

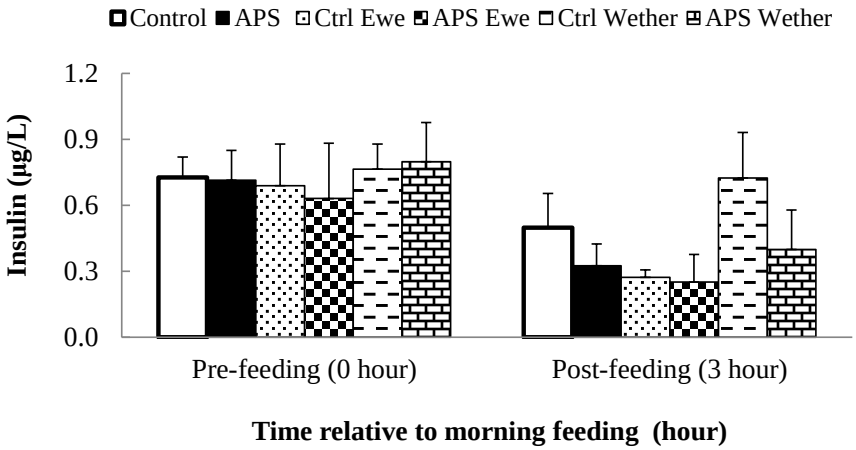
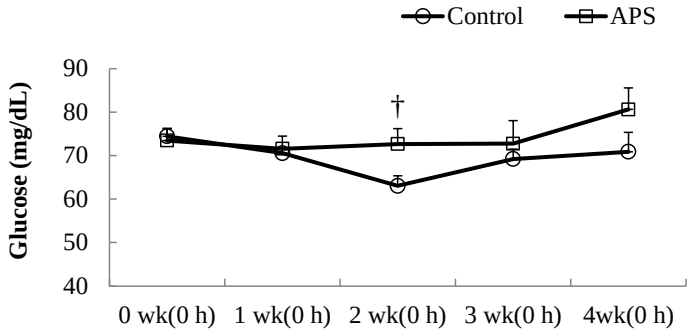


Figure 4.6: Time course changes of plasma Insulin of Suffolk sheep fed on control or APS tretment.

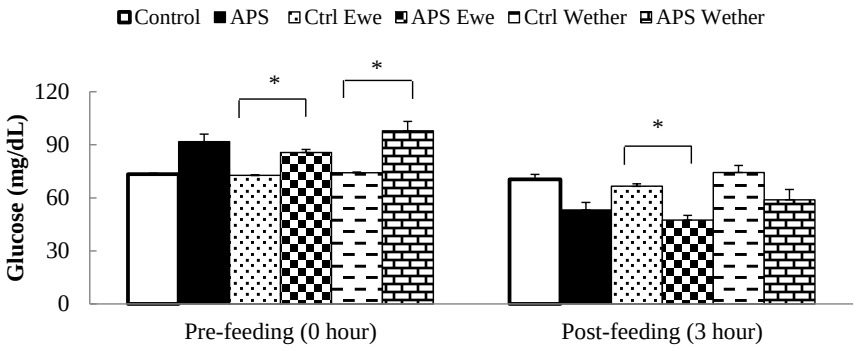
In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2).



(A) Weekly plasma concentration pattern relative to morning pre-feeding



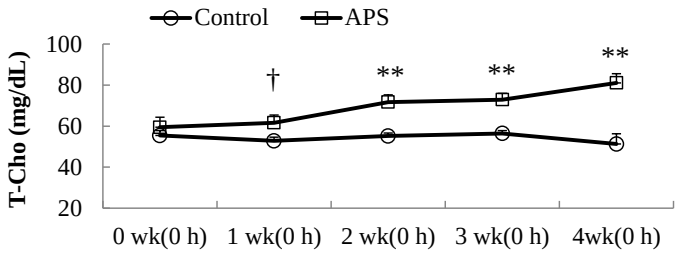
(B) Time relative to morning feeding (hour)

Figure 4.7: Time course changes of plasma Glucose of Suffolk sheep fed on control or APS tretment.

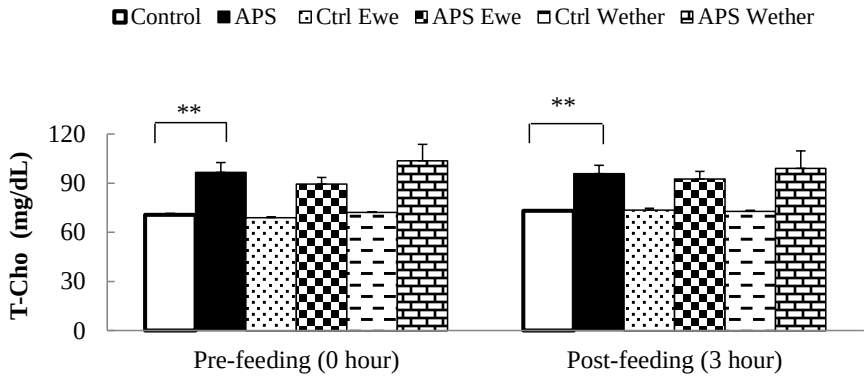
In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2). The dagger or asterisk(s) indicate a tendency toward significant difference or the significant difference (s) between treatments at each time point (†$P < 0.1$ and *$P < 0.05$).



(A) Weekly plasma concentration pattern relative to morning pre-feeding



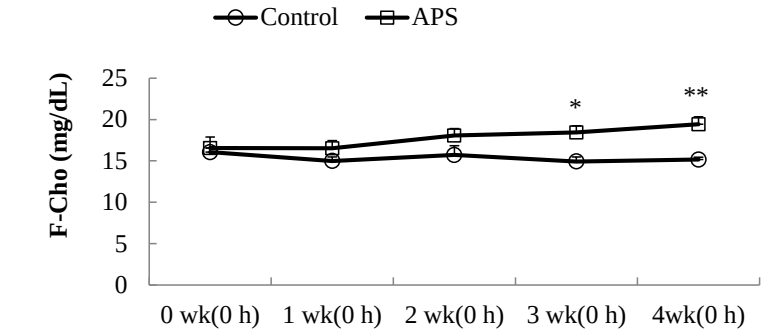
(B) Time relative to morning feeding (hour)

Figure 4.8: Time course changes of plasma Total-Cholesterol (T-Cho) of Suffolk sheep fed on control or APS tretment.

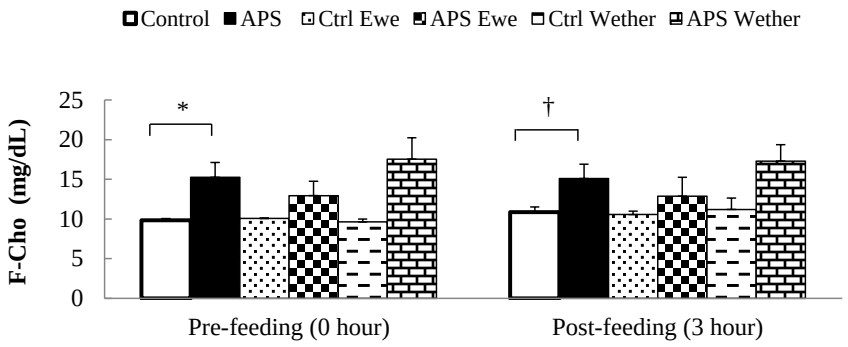
In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2). The dagger or asterisk(s) indicate a tendency toward significant difference or the significant difference (s) between treatments at each time point (†P < 0.1, *P < 0.05 and **P < 0.01).



(A) Weekly plasma concentration pattern relative to morning pre-feeding



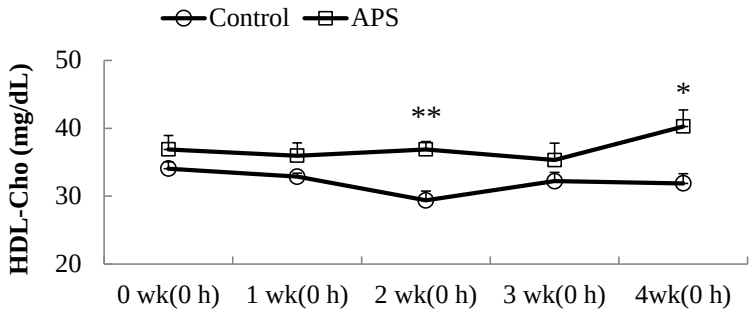
(B) Time relative to morning feeding (hour)

Figure 4.9: Time course changes of plasma Free-Cholesterol (F-Cho) of Suffolk sheep fed on control or APS tretment.

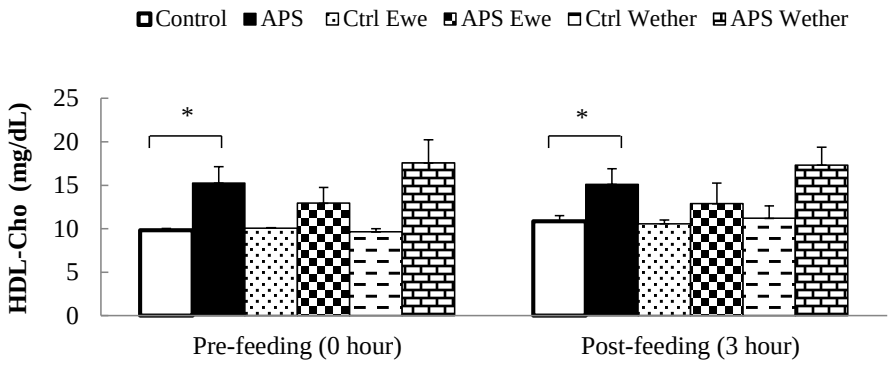
In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2). The dagger or asterisk(s) indicate a tendency toward significant difference or the significant difference (s) between treatments at each time point (†$P < 0.1$, *$P < 0.05$ and **$P < 0.01$).



(A) Weekly plasma concentration pattern relative to morning pre-feeding



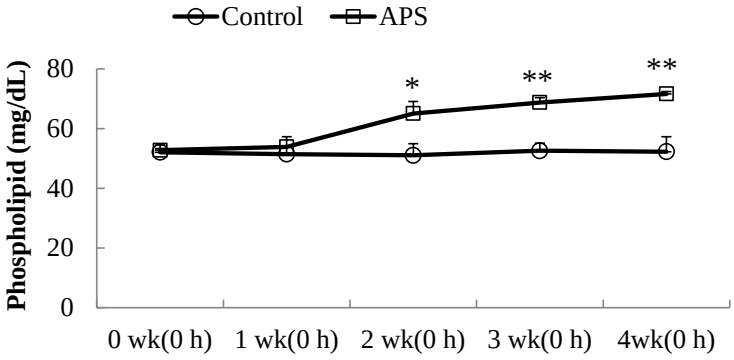
(B) Time relative to morning feeding (hour)

Figure 4.10: Time course changes of plasma high density lipoprotein cholesterol (HDL-Cho) of Suffolk sheep fed on control or APS tretment.

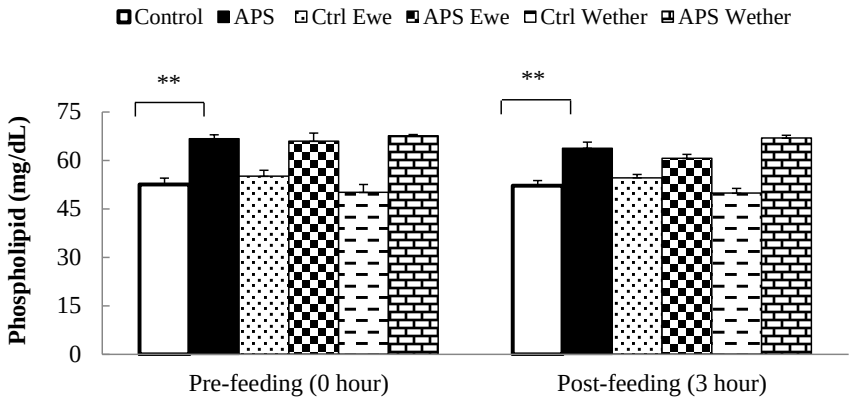
In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2). The dagger or asterisk(s) indicate a tendency toward significant difference or the significant difference (s) between treatments at each time point (*P < 0.05 and **P < 0.01).



(A) Weekly plasma concentration pattern relative to morning pre-feeding



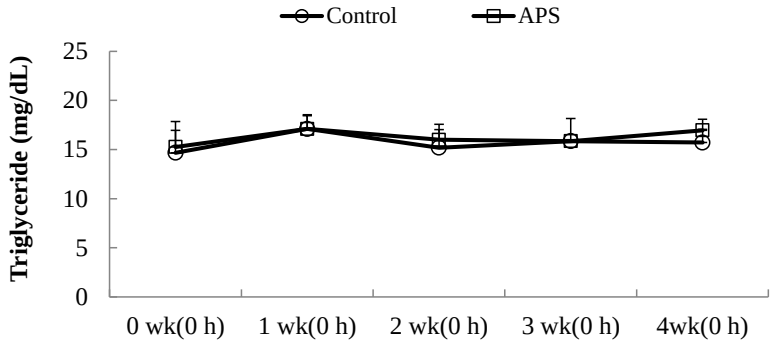
(B) Time relative to morning feeding (hour)

Figure 4.11: Time course changes of plasma Phospholipid of Suffolk sheep fed on control or APS tretment.

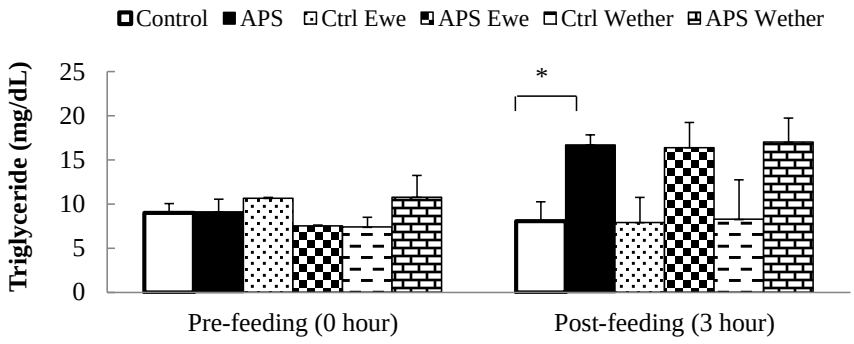
In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2). The asterisk(s) indicate the significant difference (s) between treatments at each time point (*P < 0.05 and **P < 0.01).



(A) Weekly plasma concentration pattern relative to morning pre-feeding



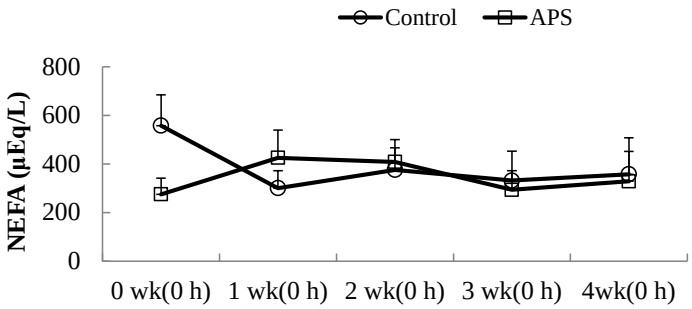
(B) Time relative to morning feeding (hour)

Figure 4.12: Time course changes of plasma Triglyceride of Suffolk sheep fed on control or APS tretment.

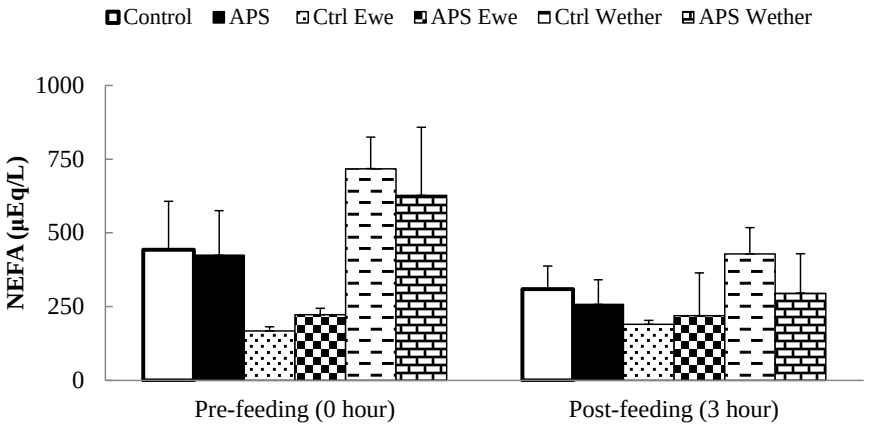
In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2). The asterisk indicate the significant difference (s) between treatments at each time point (*$P < 0.05$).



(A) Weekly plasma concentration pattern relative to morning pre-feeding



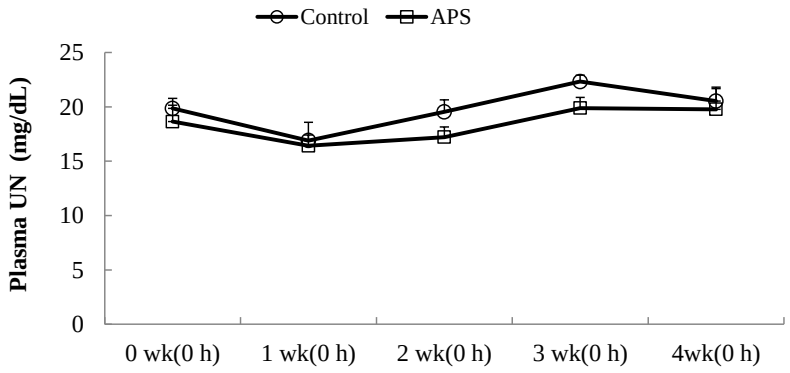
(B) Time relative to morning feeding (hour)

Figure 4.13: Time course changes of plasma non-esterified fatty acids (NEFA) of Suffolk sheep fed on control or APS tretment.

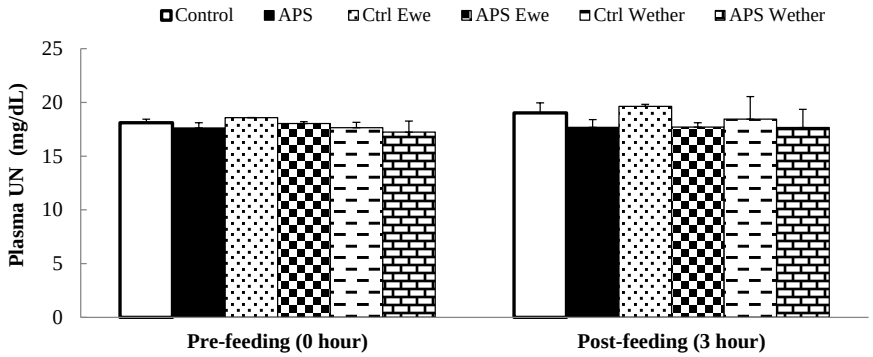
In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2).



(A) Weekly plasma concentration pattern relative to morning pre-feeding



(B) Time relative to morning feeding (hour)

Figure 4.14: Time course changes of plasma urea nitrogen (UN) of Suffolk sheep fed on control or APS tretment.

In control treatment: Control, open bar; Ewe, dotted bar; Wether, dashed bar.

In APS treatment: APS, solid bar; Ewe, diamomd bar; Wether, brick bar.

Each data point is mean ± standard error of the means (treatment, n=4; ewe, n=2; wether, n=2).

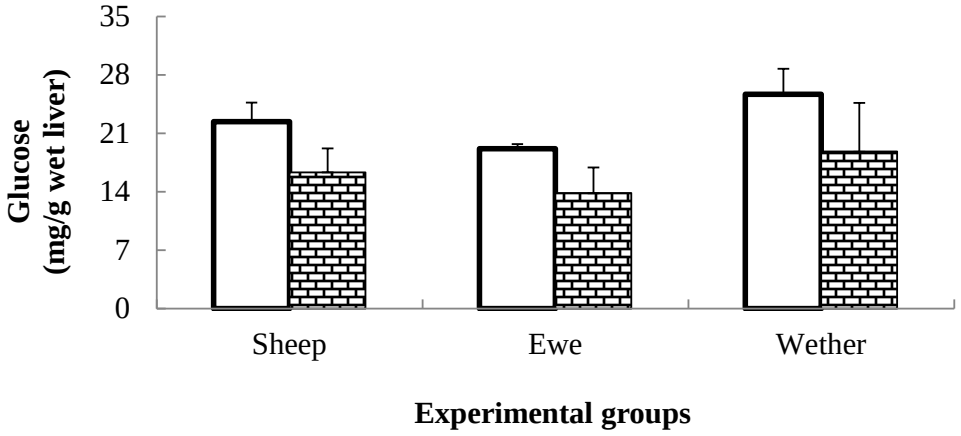


Figure 4.15: Glucose molecules of glycogen in Suffolk sheep liver fed on control (open bar) or APS (brick bar) tretment. The ethanol intake in APS treatment was calculated to be 31.4, 32.1 and 30.7 g/d/head for APS treatment (n=4), ewe (n=2) and wether (n=2), respectively. Each data point is mean ± standard error of the means.

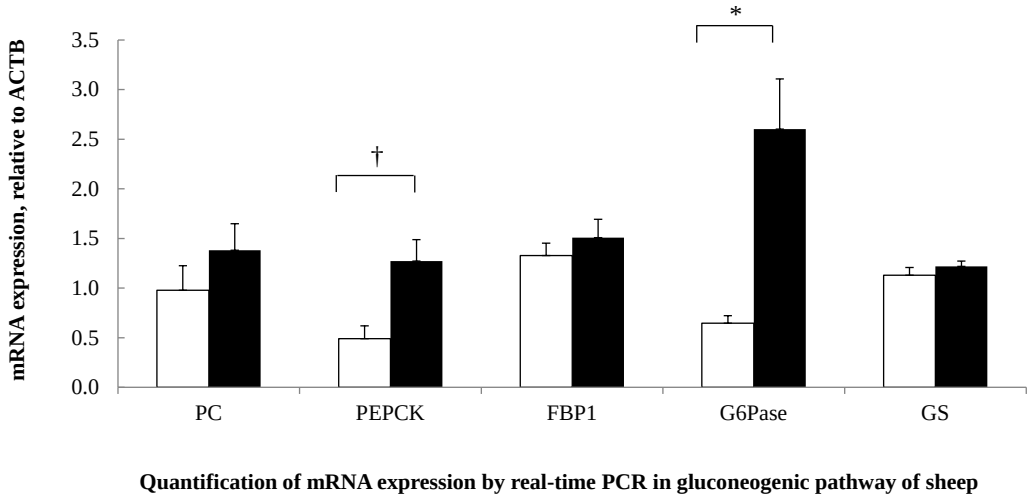


Figure 4.16: Quantification of mRNA expression by real-time PCR in gluconeogenic pathways of the Suffolk sheep fed on control (open bar) or APS (closed bar) tretment. The ethanol intake in APS treatment was calculated to be 31.4 g/d/head for treatment (n=4). Each data point is mean ± standard error of the means. The dagger or asterisk indicate a tendency toward significant difference or the significant difference between treatments ((†$P < 0.1$; *$P < 0.05$).

CHAPTER 6

General Discussion

1. Prominent production of ethanol from apple pomace silage (APS)

Fresh AP contains considerable amount of soluble sugars which can be an excellent substrate for ethanol fermentation (Hang et al., 1981). In fact in these studies, largest ethanol content was observed in fermented AP in comparison with APSs that blended with other ingredients to improve nutritional balance of AP after 2 month storage period under similar anaerobic condition. The fermented AP contained a comparable amount of ethanol (243 g kg^{-1} DM) in a single ingredient APS (173 g kg^{-1} DM) reported by Alibes et al. (1984). By using fermented AP for preparation of APS, we succeeded to increase its highest ethanol content by 228% (Chapter 4) and 148% (Chapter 3) compared to that of RS-APS (Chapter 2.1) and L-APS (Chapter 3), respectively. In these studies, ethanol content of different APSs were 33.4 g kg^{-1} for RS-APS (Chapter 2.1), 48.7 and 87.2 g kg^{-1} for L-APS/H-APS (Chapter 3), 44.1 and 66.9 g kg^{-1} for L-APS/H-APS (Chapter 2.2) and 109.4 g kg^{-1} for APS (Chapter 4). These APS contained some 2 - 8 times greater ethanol content compared to corn, grass and TMR silages (McDonald et al., 1991; Raun and Kristensen, 2010; Lawrence et al., 2011; Cummins et al., 2009).

2. Performance traits of sheep fed apple pomace silages (APSs) ethanol

A slight but significant reduction in average BWG was observed in the RS-APS than control treatment in experiment 1 (Chapter 2.1) however significantly higher BWG was observed in experiment 4 (Chapter 4). Although average BWG was also higher in experiment 2 and 3 (Chapter 2.2 and 3) for APS groups but the differences were not significant by treatment. Improved ADG and FCR by feeding of both ensiled and dried AP was also reported in finishing lambs (Taasoli and Kafilzadeh, 2008). The RS-APS used in the Chapter 2.1 had lower CP and higher fiber contents compared to

the concentrate which influence the lower growth performance in RS-APS treatment. Therefore, ingested dose of ethanol (~50 g/d) from APSs in these studies would not cause any harmful influence on performance of sheep.

3. Nutritive values of apple pomace silages (APSs) for ewes

The digestibility of DM, OM, CP, CF, EE, NFE, NDF and hemicellulose were higher in the ewes fed APSs than those of alfalfa hay diet receiving ewes. Ahn et al. (2002) reported slightly lower DM values for Korean goat with compare to in this study. DM content of APSs were higher than that of reported by others (Alibes et al., 1984; Nikolić and Jovanović, 1986; Pirmohammadi et al., 2006 and Taasoli and Kafilzadeh, 2008). The ensiling proteolysis process increases in the proportion of ammonium nitrogen and free ∝-amino acid nitrogen in the silage compared with the original material (McDonald et al., 2011) which was the reason of higher CP content in H-APS compared with L-APS. Hang et al. (1981) reported that yeast fermentation increase the amount of protein in AP by over 50% whereas in this study it was 67% for fermented AP and was 6.4% higher in H-APS compare with L-APS. Fermented APSs were a replacer of concentrate for ruminants, indeed could increase the estimated apparent CP digestibility of L-APS (74.6%) and H-APS (75.7%) and that level was slightly decreased in the APS diets (73.2 and 73.6% for L-APS and H-APS diets, respectively). However, APS diet having higher digestible CP than that of hay diet (72.3%) and fermented AP influenced slightly higher CP digestibility for H-APS diet than that of L-APS diet. On the other hand, Alibes et al. (1984) found lower estimated apparent CP digestibility for APSs (18.0-45.3%) and APS diets also (44.9-63.4%). Such difference indicate that this nutritionally balance fermented APSs are capable to overcome constrain of low protein content in AP and will able to fulfil the protein requirement of ruminants. Alibes et al. (1984) reported that when diets containing high amount of APS then the OM digestibility for APS diets were 70.1 to 77.7% and estimated OM digestibility for APSs were 74.4 to 80.4%. These estimated values are corresponded very well to the fact that ewes received 70% fresh or fermented AP containing L-

APS/H-APS and our findings were 79.6-80.9%. However, Taasoli and Kafilzadeh (2008) found slightly lower OM digestibility values 69.9 and 71.8% for ensiled and dried AP compared to these findings. Pirmohammadi et al. (2006) reported the lower ME values (9.7 and 9.0 for dried and ensiled AP) than those in this study. Moreover, Pirmohammadi et al. (2006) also reported the ME value for maize silages (10.3MJ kg^{-1} DM) which was lower than those in this study. Such difference indicate that this nutritionally balance fermented APSs are capable to overcome that constrain of low protein content in AP and will able to fulfil the protein requirement of ruminants. There was no significant difference between L-APS and H-APS for the digestibility and nutritive value of all nutrients which were calculated by difference method. No significant difference was also observed between the APSs for TDN content and ME value. Method of silage making, ensiling condition and type of feed ingredients as the absorbent, may have a great impact on the quality of silage and hence the amount that is consumed by animal. The preparation of APSs with grains by products seems to be promising, as it prevents the loss of drain fluid that are especially rich in nutrients and contains moderate concentration of fermentation end products, which is a fact of importance as far as ethanol is concerned.

4. Nitrogen balance of sheep fed apple pomace silage (APS) diets

Negative balance was not occurred during this experimental period. The APSs had more nutrient digestibility, DCP, TDN and ME values, and resulted in less nitrogen excretion in faeces and urine. The higher DM containing alfalfa hay cube intake increased the higher nitrogen intake in the hay diet. In addition, nitrogen retention efficiency was also higher in APS diets but the values were non-significant. Under such energy-sufficient condition in APSs, nitrogen utilization was higher in APSs receiving ewes which may have stimulated growth.

5. Feeding effect of apple pomace silage (APS) ethanol on response of blood parameters

In all of experiments after feeding, APS ethanol ingestion influence the ethanol load in the liver and followed by hours of elevated peripheral concentrations of ethanol. These studies revealed that dietary ethanol is highly correlated with plasma ethanol which positively influenced the plasma BHBA and lactate levels. Kristensen et al. (2007) also observed that after feeding of corn silages plasma concentrations of ethanol, lactate and BHBA were increases. Nonetheless, all of these blood parameters determined in this study returned to pre-feeding levels until 8 hours after morning feed and none of clinical signs of health problem was observed. As Alibes et al. (1984) cautioned against overfeeding of alcoholic fermented APS, ethanol content of APSs used in the present study was more than at least five times as much as corn silage (McDonald et al., 1991; Kristensen et al., 2007; Raun and Kristensen, 2010). Increased dose of ethanol in experiment 4 (Chapter 4) produced higher plasma cholesterols, phospholipid and triglyceride as well as fat accumulation in the liver. This is probably because of ethanol intake in the APS treatment was within the aforementioned metabolizable range. It is established that ethanol inhibit hepatic gluconeogenesis from lactate, glycerol, some amino acids and other substrates (Krebs et al., 1969) and thus cause postprandial hypoglycemia in sheep fed on APS diets (Kondo et al., 2010; 2011) and reduced postprandial hypoglycemia in human subjected to consumed alcoholic beverages with a meal (Brand-Miller et al., 2007). After ingestion of APS ethanol, plasma glucose concentrations were declined in the Chapter 3 and Chapter 4 which indicated that ethanol ingestion inhibit the gluconeogenesis and glucose output. However, elevated plasma lactate and BHBA levels should be an indication of suppressed gluconeogenesis. The AUC of plasma ethanol and BHBA for Chapter 2.1 and 3 were grater and the negative correlations between plasma glucose AUC and the AUCs of plasma lactate and BHBA in Chapter 4 may also reflected ethanol-suppressed gluconeogenesis and resultant hypoglycemia in APS consumed animals. Veenhuizen, et al. (1991) also reported that hepatic in vitro gluconeogenic capacity decreased significantly for ketosis cow and at kitotic stage plasma BHBA increased 3.5~8.4 fold by using feed restriction plus dietary 1,3-butanediol. In the fact the sheep consumed

APSs ethanol and plasma BHBA increased dramatically 4.5~9 fold for peaked hour which indicated that ketosis occurred in these studies. Thus, all of hypoglycaemic appearance was occurred in ewes, this ethanol-induced hypoglycaemia would conceivably be greater for ethanol ingested ewes compared to wether. Well-fed animals like growing ewes in the present study would have sufficient precursors for ketogenesis. Therefore once ingested ethanol suppressed gluconeogenesis, lactate would become redundant in gluconeogenic pathway and be accumulated, and BHBA would also be generated from fatty acids in well-fed ruminant animals. Consistent positive correlations between the AUCs of plasma lactate and BHBA concentrations in both Chapter 2.1 and 2.2; the negative correlations between the AUCs of plasma glucose and BHBA in both Chapter 2.2 and 3; positive correlations between the AUCs of plasma ethanol and triglyceride concentrations in Chapter 3 and positive correlations between the AUCs of plasma BHBA and triglyceride concentrations in Chapter 2.2 are strongly supporting this notion.

6. Feeding effect of apple pomace silage (APS) ethanol on hepatic tissue

Liver weight was increased in APS receiving group than control. The elevated fat composition of the APS receiving group could be a factor for the increase in liver weight. Sumida et al. (2005) suggested that an interaction between the high fat content of the diet and ethanol, which could contribute to the elevation in liver weight. In support, APS contained higher amount of dietary fat compare to control. The inhibitory effect of APS ethanol upon hepatic gluconeogenesis resulting in lower glucose output in the blood (Chapter 2.2, 3 and 4) which may lead to 27% lower glucose concentration in the liver (Chapter 4).

After feeding of APS diets, the mRNA expression of both key enzymes (PEPCK and G6Pase) were tended to increase or increased (2.5 and 4.2-fold greater, respectively) in APS receiving sheep liver. This study is the first to report that mRNA expression increases for rate-limiting gluconeogenic enzymes of PEPCK and G6Pase by feeding alcoholic fermented APS through suppression of hepatic gluconeogenesis. Li et al.

(2013) also observed higher or tended to be higher of PEPCK and G6Pase mRNA expression for corn silages receiving group, however they were not considered of silage ethanol whereas another group received without corn silage diet and they concluded that different dietary ingredients could change gene expression of hepatic gluconeogenesis pathway. Pilkis and Granner (1992) also reported hepatic PEPCK activity is markedly increased in fasted or diabetic animals. Consistent plasma glucose declined at 2-3 hours after morning feed for APS group in the previous experiments (Chapter 2 and 3) and postprandial glycaemia at 3 hour after morning feed for H-APS receiving ewes of this experiment are strongly supporting this notion. Kondo et al. (2010; 2011) also reported the postprandial lower plasma glucose concentration due to ingestion of APS ethanol. Thorn, et al. (2012) reported that hepatic gluconeogenic genes (PEPCK, G6Pase, FBP1, GLU2 and PGCIA) expression increased in the hypoglycemic sheep fetuses induced by maternal insulin infusion for 8 weeks which are correspond very well to our observations. However we have failed to detected the significant increase of FBP1 for APS receiving sheep liver. PC expression was 1.4-fold greater in the alcoholic sheep liver however no significant effect was detected. GS was not influence by the APS ethanol which indicates that APS ethanol does not affect hepatic glycogen content. Rozance et al. (2008) also reported that hepatic PEPCK and G6Pase mRNA increased 12-fold and 7-fold, respectively, following chronic hypoglycaemia with no change in hepatic glycogen. This statement is correspond very well to this present study, however our findings were somewhat smaller. Therefore, our study demonstrated that in order to respond of APS ethanol, genes involved in gluconeogenesis pathway played an important role and fermented APS feeding have a positive influence on the activity of key gluconeogenesis enzymes in alcoholic sheep. We speculated that PEPCK and G6Pase might be the most vital regulatory genes in hepatic gluconeogenesis of sheep liver for ethanol metabolism.

After feeding of APSs, plasma ethanol and BHBA concentration were increased however we failed to detect ethanol induce liver injury biomarker GOT and GPT toxic

effect. Moreover, liver glycogen and lipid content were not influenced by the APS treatments and no clinical symptom was observed.

7. Conclusion

In this study, it is confirmed that apple pomace can be well preserved by ethanol fermentation and nutritionally balanced APS can be used as a replacer of concentrate feed for ruminants. Suffolk sheep are capable to utilize ~50 g/d ethanol intake from APS without any clinical sign of adverse effect.

Examination of response in blood parameters after feeding of APS ethanol revealed increase in plasma ethanol, BHBA and lipid profiles and hypoglycaemic tendency in ewes for APS receiving group, it would be concluded that APS ethanol consumption induce postprandial transient hyperketonemia through suppression of hepatic gluconeogenesis and the ethanol-induced suppression of gluconeogenesis was greater in ewes. Heavier liver and higher mRNA expression for PEPCK and G6Pase were observed after APS ethanol ingestion which point out that PEPCK and G6Pase might be the most vital regulatory genes in hepatic gluconeogenesis of sheep liver for ethanol metabolism.

These results indicate that APS ethanol consumption induce postprandial transient hyperketonemia through suppression of hepatic gluconeogenesis while the liver has to be giving priority to metabolize ethanol over other hepatic metabolism. Therefore, farmers can use the fermented APS for ruminant feeds as a replacer of concentrate feed but need to be cautious when they fed alcoholic fermented feeds to ruminant livestock.

CHAPTER 7

Summery and implications

Ethanol is a common constituent of fermented feeds for ruminant livestock. Apple pomace contains high amounts of moisture, fermentable carbohydrate and organic acids which are responsible for prominent production of ethanol in apple pomace silage (APS). Excess alcohol intake lead to an increased alcohol load into liver and followed by a period of elevated peripheral concentrations of alcohol, causing changes in ruminal fermentation pattern and metabolite concentrations in blood, and may influence the postprandial metabolism. Thus, this study was undertaken to investigate the effect of feeding APS which is rich in ethanol on blood parameters, liver glycogen and some regulatory enzymes for hepatic gluconeogenesis. Nutritive values of APS containing different amounts of ethanol were also evaluated in Suffolk sheep.

Chapter 1. Effect of feeding apple pomace silage (APS) ethanol on response of blood parameters in Suffolk ewes

Two experiments were conducted. In experiment 1, four ewes were fed alfalfa hay cube and either rice straw APS (RS-APS, 33.4 g of ethanol/kg DM) or concentrate (control) in a 2×2 crossover design over two 35-day periods. Alfalfa hay cube and either the RS-APS or concentrate were provided at a ratio of 30:70 of TDN requirement for maintenance and daily 100g gain. On the last day of each 35 days period, blood samples were collected during 8 hours before and after morning feed, and plasma levels of ethanol, lactate, β-hydroxybutyrate (BHBA), insulin, metabolites, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were assayed. The area upper or under the curve (AUC) of blood parameters for 2 and 8 hours were calculated and assessed correlations among the AUCs.

Plasma concentrations of ethanol, lactate and BHBA were elevated after feeding of RS-APS and peaked at 2, 1 and 4 hours, respectively. Plasma insulin level tended to increase after RS-APS feeding but the magnitude of postprandial glucose decline was

not different between the treatments. After feeding of RS-APS, plasma F-Cho, HDL-Cho and phospholipid were increased and plasma urea nitrogen (UN) was decreased. The AUCs of plasma ethanol and lactate were positively correlated with that of BHBA. Insulin AUC was positively correlated with AUCs of ethanol and BHBA. The AUCs of plasma GPT was positively correlated with that of BHBA.

In experiment 2, effects of feeding low and high levels of ethanol (44.1g vs. 66.9 g of ethanol/kg DM) contained in two APS (L-APS and H-APS) on blood parameters were compared. Animals, design, feeding level, duration, sampling procedure and parameters tested were same as in experiment 1.

The changes in blood parameters showed similar trends after feeding of two APSs, which correspond to the changes after APS feeding in experiment 1, however no significant treatment effect was detected. The AUC of lactate was positively correlated with those of BHBA and triglyceride. The BHBA AUC was also positively correlated with that of triglyceride. The glucose AUC was negatively correlated with AUCs of lactate and BHBA.

Simultaneous increases in plasma ethanol, lactate and BHBA, and the correlations between these AUCs would indicate that higher priority of ethanol metabolism are given over glucose metabolism in the liver after APS ethanol consumption.

Chapter 2. Nutritive values of apple pomace silages containing different amounts of ethanol in Suffolk ewes

Ethanol production during ensiling leads to larger DM loses compared to common lactic acid fermentation. Therefore ethanol content may affect nutritive values of APS containing different amounts of ethanol. In this experiment, 3 ewes were used in a 3×3 Latin square design over three period assigned with three dietary treatments: hay diet, L-APS (48.7 g of ethanol/kg DM) diet and H-APS (87.2 g of ethanol/kg DM) diet. Alfalfa hay cube and either the L-APS or H-APS provided half of 110% TDN requirement for maintenance in L-APS or H-APS diet and control group fulfil 110%

TDN requirement from alfalfa hay cube. During the last 7 days of each 21 day period, feaces, urine and feed refusal were collected. Blood samples were collected during 3 hours before and after morning feed, and plasma levels of ethanol, lactate, β-hydroxybutyrate (BHBA), metabolites and glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were assayed. The area upper or under the curve (AUC) of blood parameters for 3 hours were calculated and assessed correlations among the AUCs.

Apparent digestibility of all nutrients were higher in APS diets and CP digestibility was lower in L-APS diet. There was no significant difference in nutrient digestibility of all items and TDN content between the L-APS and H-APS. Nitrogen intake, faecal nitrogen excretion and total nitrogen excretion were higher in ewes fed hay diet and there was no significant different between the both APS diets. Higher ethanol intake produced higher plasma ethanol concentration in the H-APS than L-APS treatment. Postprandial changes in plasma parameters after feeding of two APSs followed similar trends in the experiment 1 and 2, and the magnitude of response was not different between L-APS and H-APS treatments. Based on the overall findings, it is indicated that AP can be well preserved by ethanol fermentation and nutritionally balanced APS can be used as a replacer of concentrate feed for ruminants in combination with hay cube or dry roughage up to 50% of the TDN requirement. Therefore, ingestion does of ethanol (~15.1 g/d) from APSs in this study would not cause any harmful influence on performance of ewes.

Chapter 3. Effect of ethanol contained in apple pomace silage on hepatic metabolism of Suffolk sheep

Four each of ewes and wether were fed alfalfa hay cube and either APS (109.4 g of ethanol/kg DM) or concentrate (control) in a 2×2 factorial design over 28 day periods. Alfalfa hay cube and either APS or concentrate were provided at a ratio of 30:70 of TDN requirement for 110% of maintenance. Blood samples were collected weekly immediately before morning feed, and at exactly after 3 hours of morning feed

immediately before slaughtering on the last day of 28 day period. All plasma samples were assayed for ethanol, lactate, BHBA, insulin, plasma metabolites, GOT and GPT. The animal was slaughtered after 3 hours of morning feed, and liver sample was immediately collected for measurements for glycogen content and mRNA expression for the genes related gluconeogenesis; pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBP1), glucose-6-phosohatase (G6Pase), glycogen synthase (GS). Whole blood and internal organs weight were recorded.

In APS group, pre-feeding plasma levels of ethanol, glucose, T-Cho, F-Cho, HDL-Cho, and phospholipid were increased and that of BHBA was declined. Postprandial plasma ethanol, BHBA, T-Cho, F-Cho, HDL-Cho, phospholipid and triglyceride concentrations were increased in APS group and postprandial glucose concentration was not affected by treatment however hypoglycemic tendency occurred in ewes. APS group had significant heavier liver. The mRNA expression of PEPCK tended to greater and G6Pase was significantly greater in the liver from H-APS sheep, presumably because of compensatory over expression of these gluconeogenic enzymes under a condition of suppressed gluconeogenesis generated by ethanol consumption from APS.

In this study, it is confirmed that apple pomace can be well preserved by ethanol fermentation and nutritionally balanced APS can be used as a replacer of concentrate feed for ruminants. Suffolk sheep are capable to utilize ~50 g/d ethanol intake from APS without any clinical sign of adverse effect. Examination of responses in blood parameters after feeding of APS ethanol revealed increased plasma ethanol, BHBA and lipid components. Heavier liver and higher mRNA expression for PEPCK and G6Pase were observed after APS ethanol ingestion. These results indicate that APS ethanol consumption induce postprandial transient hyperketonemia through suppression of hepatic gluconeogenesis while the liver has to be giving priority to metabolize ethanol over other hepatic metabolism. Therefore, farmers need to be cautious when they fed alcoholic fermented feeds to ruminant livestock.

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