Studies on whole crop rice total mixed ration silage preparation with food by-products and its feed characteristics

by

Yang CAO

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Iwate University

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Abbreviations

A:P : Acetic acid to propionic acid ratios

ADF : Acid detergent fiber

BW : Body weight

$BW^{0.75}$: Metabolic body weight

CH_4 : Methane

CO_2 : Carbon dioxide

CP : Crude protein

DDM : Digestible dry matter

DE : Digestible energy

DM : Dry matter

DMI : Dry matter intake

DTC : Dry tofu cake

EE : Ether extract

FJLB : Fermented juice of epiphytic lactic acid bacteria

FM : Fresh matter

FTMR : Fermented total mixed ration

GE : Gross energy

LAB : Lactic acid bacteria

LAC : Lactic acid content

NAD : Nicotinamide adenine dinucleotide

NADH : Reduced form of NAD

NDF : Neutral detergent fiber

NFC : Nonfibrous carbohydrate

NFE : Nitrogen free extract

NH_3-N : Ammonia nitrogen

OM : Organic matter

RB : Rice bran

SEM : Standard error of the mean

TDN : Total digestible nutrients

TMR : Total mixed ration

TN : Total nitrogen

VFA : Volatile fatty acids

Vs. : Versus

WCR : Whole crop rice

WCRS : Whole crop rice silage

Chapter 1

General Introduction

1.1. Fermented total mixed ration

A total mixed ration (TMR) is a single feed mix for cows that combines all forages, grains, protein feeds, minerals, vitamins, and feed additives formulated to a specified nutrient concentration. Fermented TMR has a moderate amount of moisture, a higher lactic acid content (LAC), and produces high-quality TMR silage.

1.1.1. Using whole crop rice and food by-products to prepare TMR silage

At present, roughly 63 hundred million people live on Earth, and about 8 hundred million suffer from nutrient deficiency or starvation (Ishibashi, 2007). Food self-sufficiency in Japan is 40% (calorie conversion), much lower than that of Australia (230%), France (130%), the United States (119%), Germany (91%), the United Kingdom (74%), and 167 other nations in the world (Ishibashi, 2007). As the production of livestock has increased, and with it the consumption of grains and other crops, competition for food has intensified. Because the animal feed-sufficiency of Japan is 24.7% (total digestible nutrient (TDN) conversion; Ishibashi, 2007), it is important to improve feed self-sufficiency. Although the output of assorted feed in Japan has been about 24 million tons in recent years, with a consumption extension of animal products, increasing the production of feed is also necessary. However, even most of the raw materials used for feed production are currently imported from foreign countries. Since

the bovine spongiform encephalopathy ("mad cow disease") epidemic, the interest in feed safety has heightened. Therefore, it has become more important than ever to develop a safe and robust feed self-sufficiency program in countries like Japan (Warren and Farrell, 1990; Aguilera et al., 1992; Gatel, 1994; Hatano et al., 2002; Struble and Aomari, 2003; Sato, 2005; Suzuki and Kaiser, 2005). The domestic production of feed for domestically raised animals is an obvious way to improve low feed self-sufficiency rates (Ogino et al., 2008).

Over the past several years, Japan has experienced a decrease in human rice consumption, while the grain's importance for feed has increased. Whole crop rice (WCR) is a suitable feed crop because it can withstand the high heat and rain of Japanese summers. There is an increasing level of research interest in Japan on the utilization of WCR as a feed for ruminants, because more than 400,000 ha of paddy fields are currently unused due to production adjustments (Shioya and Cai, 2004; Nishino et al., 2007). To increase the production of domestic roughage in Japan, forage rice plant crop acreage was recently increased to more than 5,000 ha (Tobisa et al., 2005), and forage rice for ratoon cropping could one day be cultivated in the Kyushu region (Kobayashi et al., 2007). Experts have developed special harvesting machines as well as species adapted to local cultivation techniques and conditions. However, WCR silage is a poor-quality feed due to low LAC resulting from a low soluble carbohydrate content (Yoshida et al., 1987; Enishi and Shijimaya, 1998; Cai et al., 2003). In addition, producing high-quality self-sufficiency roughage requires the development of a robust breed as well as efficient culturing, harvesting, and preparation techniques (Enishi and Shijimaya, 1998; Islam et al., 2001, 2004; Cai et al., 2003; Urakawa et al., 2004; Matsuyama et al., 2005, 2006; Tsuru et al., 2007).

The processing and manufacturing of many foods and beverages produce large quantities of edible byproducts, and the amount has increased remarkably in recent years. In Japan, ca. 11,314,000 tons of food by-products were generated in 2002. However, only about 1.6% (182,000 tons) was used as feed and 1.65% (187,000 tons) as compost, while about 45% (5,060,000 tons) was recycled. Most of it was burned or dumped into landfills (Xu and Toyokawa, 2005). This appears to be a waste of potential resources and energy. However, demand is increasing for the efficient use of food by-products due to economic and environmental concerns, and byproducts such as tofu cake (TC), rice bran (RB), and wet green tea waste (WGTW) are high in crude protein, fatty acid, tannins, and vitamins (Xu et al., 2001, 2007; Amissah et al., 2003; Kondo et al., 2004a; de Campos et al., 2007). In Japan, more than 700,000 tons of TC, 529,200 tons of RB, and 100,000 tons of WGTW are produced annually (Kajikawa, 1996; Xu et al., 2001; Kondo et al., 2004a). Not only could these byproducts be used as a source of nutrients for ruminants, but using them to replace imported commercial feedstuffs could save energy on transporting imports and possibly reduce the environmental impact of burning them as waste or burying them in a landfill (Furumoto, 2002; Ide, 2002; Ishida, 2002).

Ensiling WCR with these food by-products into a TMR could potentially produce good-quality silage with high LAC. Unpalatable byproducts could also possibly be incorporated into the TMR if their odors and flavors were altered by fermentation.

1.1.2. Quality and conservation of fermented TMR

The quality of round-baled TMR silage differs depending on the source TMR used. For example, Hiraoka et al. (2005) found that the dry matter loss rate was 10% and 3.4% after 24 h under aerobic conditions for fresh and fermented TMR, respectively. In

addition, mold proliferated with time in fresh TMR but not fermented TMR, which had a fresh matter (FM) LAC of 4.3% and a pH of 4. Good fermentation quality facilitates high-density roll baling of chopped material (Shito and Yamana, 2002; 2003). In addition, TMR can be further improved by using a strong vinyl bag inside a transbag during fermentation to increase de-aeration. Shioya (2008) combined a roll baler and a transbag to produce a high-quality TMR silage with an FM LAC of 1.9–3.5% and a pH of 4.0–4.5. The preservation of TMR prepared in a transbag is similar to corn silage prepared by roll baler (Nishino et al., 2004; Shioya et al., 2006). Resistance to deterioration after opening such TMR silage is affected by its microbial flora (Wang and Nishino, 2008 a; b; in press).

1.1.3. Advantages of fermented TMR

Shioya (2008) found that cattle ate on average 4.3 kg of feed made from fermented TMR compared to 3.8 kg for fresh TMR; they also gained more body weight and had lower free fatty acid levels in their blood on fermented TMR than fresh TMR. Yamamoto et al. (2005) and Shioya (2008) compared WCR silage made from fresh and fermented TMR, and the effects of each on cows in summer. Both studies reported no significant differences in the chemical compositions and TDNs of fresh and fermented TMR, and both feeds resulted in normal rumen NH_3-N and blood urea nitrogen levels; however, fermented TMR had a higher LAC, lower nonfibrous carbohydrate content, lower overall nonfibrous carbohydrate digestibility, and significantly higher propionic acid levels but a significantly lower acetic acid:propionic acid ratio (A/P), which could be attributed to the higher LAC of fermented TMR than fresh TMR.

Some food by-products such as TC, RB, and WGTW contain protein, fatty acid, and

functional components such as catechins and vitamins that in theory could be used as feed for ruminants (Belyea et al., 1989; Arosemena et al., 1995; Cai et al., 2001; Niwa, 2001; Imai, 2002; Enishi et al., 2005). However, due to other components, such as tannins in WGTW, simply mixing these byproducts with feed results in a low-quality TMR (Reed, 1995; Eruden et al., 2005). Such mixes can be improved by using fermented TMR. Eruden et al. (2007) found that lactating dairy cows exhibited equal preference for TMR without byproducts and TMR with up to 10% WGTW. Other byproducts can also be effectively used for farm animals (Shioya, 2008).

1.1.4. Using fermented TMR for self-sufficient feed

Livestock provide about 10% of the protein and 30% of the calcium needs of people in Japan (Shioya et al., 2007), and the livestock industry also offers other resources such as animal excreta (Kano et al., 2000a,b), jobs in farming villages, and the efficient use of land via self-sufficient forage production (Shioya et al., 2007). To break the country's dependence on imported feed, it is necessary to develop a robust system of producing self-sufficient feed. Some ways this could be achieved include using idle paddy fields to produce WCR, extending domestic rice straw production, expanding grazing into abandoned farm fields, and developing the use of fermented TMR (Shioya et al., 2007). However, fermented TMR currently costs more than fresh TMR (Shioya, 2008), due to mixer and labor fees associated with the former. To lower this cost, fermented TMR could be prepared using self-sufficient forage feed such as WCR in combination with food by-products. Due to the various benefits of fermented TMR, as described in detail above, this method is receiving strong interest for the production of new varieties of TMR silage.

In general, TMR feed provides ruminants a stable, nutrient-balanced ration, thereby

facilitating good production and health. Selective consumption is minimized, and accordingly, so is the risk of digestive upsets, as rumen pH is stabilized and digestion optimized (Maltz et al., 1992). In addition, TMR mixes reduce the work of feeding cows and therefore save labor costs; provide more control over and thus accuracy in the amount of feed used; and make it easier to monitor the daily feed intake of cows and ensure that nutrient specifications are met, which can increase milk production (Maltz et al., 1992). Moreover, the high content of volatile fatty acid (VFA) in fermented TMR, better rumen fermentation, and balance of nutrients leads to improved milk fat and other important livestock products (Orozco-Hernanoez et al., 1995; Thomas et al., 2001; Miron et al., 2007).

1.2. Contribution of ruminant-produced methane to global warming

In ruminants, fermentative digestion by ruminal microorganisms produces not only energy and protein for tissue metabolism but also methane, carbon dioxide, and ammonia. Methane production is an energetically wasteful process, because the gas must be eructated from the rumen. The energy lost to methane production ranges from 2 to 12% (Johnson and Ward, 1996; Giger-Reverdin and Sauvant, 2000). Furthermore, methane emission by ruminants is considered the single largest source of anthropogenic methane and the second most important greenhouse gas (Mathison et al., 1998). Consequently, it is necessary to understand the factors that influence enteric methane production. Doing so would not only reduce uncertainty about the contribution of methane to greenhouse gas emissions and help develop viable greenhouse gas reduction strategies, but also provide an economic benefit by leading to greater energy-use efficiency of the feed. There is increased worldwide interest in mitigating the impact of ruminant-produced methane.

1.2.1. Contribution of methane to the greenhouse effect

While carbon dioxide (CO_2) receives the most attention as a factor in global warming, there are other gases to consider, including methane, nitrous oxide (N_2O), and chlorofluorocarbons (CFCs; Moss, et al., 2000). The warming role of methane in the atmosphere has been known since the 1940s, when Migeotte (1948) observed strong absorption bands in the infra-red region of the solar spectrum attributable to the presence of atmospheric methane. Like CO_2, methane traps outgoing terrestrial infrared radiation, but it does so 20 times more effectively than CO_2 (Johnson and Johnson, 1995). In addition, it absorbs radiation at wavelengths of 8–13 μm (Takahashi, 2006), and therefore even a small increase in atmospheric methane concentration has an extremely large impact on global warming. According to the Intergovernmental Panel on Climate Change (IPCC, 1994), annual methane emissions are about 535 Tg, 85 Tg of which is produced by ruminants. As the world population continues to grow, so does the demand for meat, milk, and other products of livestock, leading to more ruminant-produced methane.

1.2.2. Methane production in the rumen

Ruminants and so-called pseudo-ruminants (e.g., Camelidae, some birds) have large anaerobic fermentation chambers at both ends of their digestive tract, which greatly facilitate the digestion of carbohydrates and plant cell walls. Furthermore, microbial proteins synthesized in the fore-stomach are also digested in the small intestine, and provide up to more than 50% of the amino acids entering the blood stream (Moss et al., 2000).

Fermentation of glucose equivalents released from plant polymers or starch is an

oxidative process under anaerobic conditions that occurs in the Embden–Meyerhof–Parnas Pathway. It reduces cofactors such as nicotinamide adenine dinucleotide reduced (NADH) (Fig. 1), which then must be re-oxidized to NAD to complete sugar fermentation. NAD^+ is regenerated by electron transfer to acceptors other than oxygen (CO_2, sulfate, nitrate, fumarate). If the required cofactors are present, electron transport-linked phosphorylation inside microbial bodies generates adenosine triphosphate (ATP) via the flow of electrons through membranes (Erfle et al., 1986). The production of H_2 is a thermodynamically unfavorable process controlled by electron carriers (Wolin, 1979). Even traces of H_2 inhibit hydrogenase activity, but more H_2 is tolerated if bacteria have ferridoxin-linked pyruvate oxidoreductases (Miller and Wolin, 1973). Although H_2 is a major end product of fermentation by protozoa, fungi, and pure monocultures of some bacteria, it does not accumulate in the rumen because it is immediately used by other bacteria in the mixed microbial ecosystem. Some physical associations between fermentative species and H_2-utilizing species may facilitate interspecies transfer in the rumen, such as the attachment of methanogens to the external pellicles of protozoa (Stumm et al., 1982). In the rumen, methane formation is the major route of hydrogen elimination, which takes place via the following reaction (Moss et al., 2000):

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O$$

The transfer of hydrogen to methanogens helps degrade the cell wall carbohydrates of bacteria, fungi, and protozoa (Bauchop and Mountfort, 1981; Ushida and Jouany, 1996), a process that has been confirmed *in vivo* in gnotoxenic lambs with or without methanogens (Fonty et al., 1997).

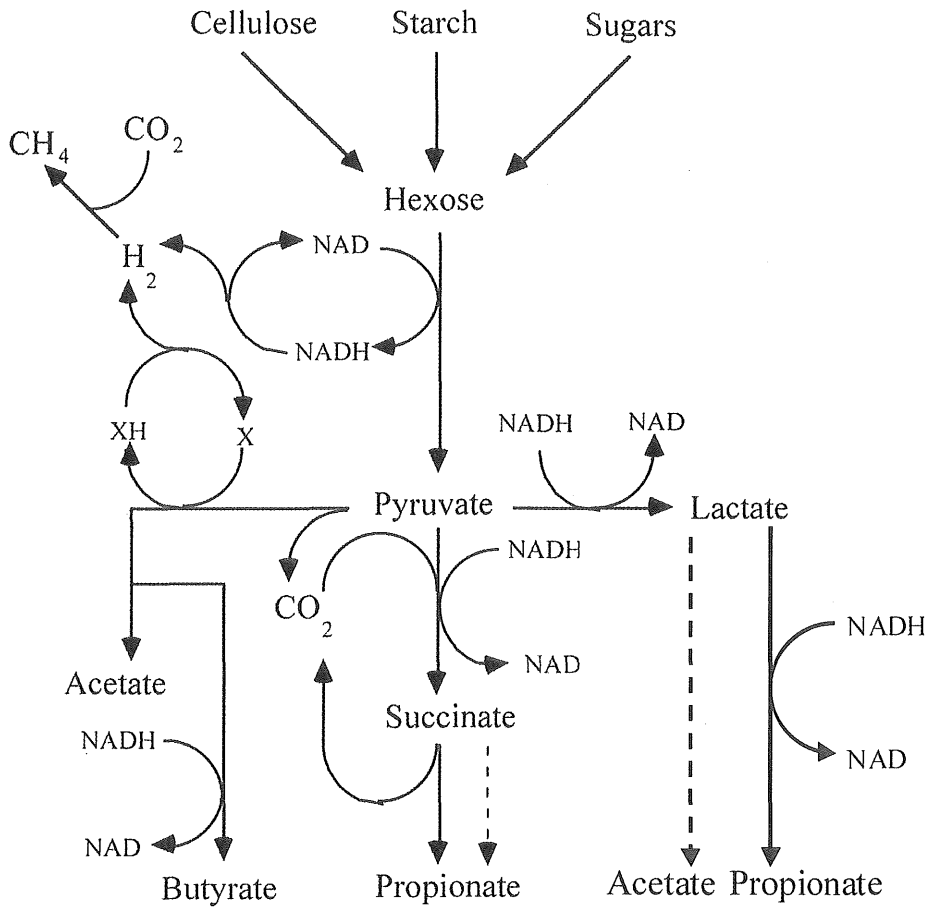

Figure 1.1. Schematic drawing showing the major pathways of carbohydrate fermentation by ruminal bacteria, where X is an alternative electron carrier (e.g., ferredoxin). In some ruminal bacteria, pyruvate decarboxylation is coupled to produce formate, most of which is converted to hydrogen and carbon dioxide by hydrogen formate lyase. The dashed lines represent pathways that occur in other organisms. (Adapted from Russel and Houlihan, 2003).

1.2.3. Propionate production in the rumen

Metabolic hydrogen in the form of reduced protons can also be used during the synthesis of VFA or incorporated into microbial organic matter (Fig. 1.1). The stoichiometry of the main anaerobic fermentation pathways can be summarized as

follows (Moss et al., 2000):

H_2-producing reactions:

$$\text{Glucose} \rightarrow 2 \text{ pyruvate} + 2H_2$$

$$\text{Pyruvate} + H_2O \rightarrow \text{acetate (C2)} + CO_2 + H_2$$

H_2-utilizing reactions:

$$\text{Pyruvate} + 2H_2 \rightarrow \text{propionate (C3)} + H_2O$$

$$\text{Lactate} + H_2 \rightarrow \text{propionate (C3)} + H_2O$$

$$2 \text{ acetate (C2)} + 2H_2 \rightarrow \text{butyrate (C4)} + 2H_2O$$

The molar percentage of VFA influences the production of methane in the rumen (Moss et al., 2000). Specifically, acetate and butyrate promote methane production while propionate formation is considered a competitive pathway for hydrogen use in the rumen. Van Kessel and Russell (1996), using rumen fluid sampled from animals fed on roughage-based diets, showed that ruminal methanogens lost the ability to use H_2 at a low pH, giving rise to free H_2 in the gas phase when pH was less than 5.5. Accordingly, in animals on roughage diets, a low pH leads to a decrease in methanogenesis independent of propionate formation. In contrast, starch-fermenting bacteria can compete with methanogens for H_2 use by producing large amounts of propionate; however, H_2 accumulates and propionate decreases dramatically while acetate increases when the pH reaches non-physiological values below 5.3 (Russell, 1998). This indicates that the microbial ecosystem involved in propionate formation differs according to dietary conditions. The cellulolytic bacteria *Fibrobacter succinogenes* is the major propionate producer via the succinate pathway in animals fed roughage diets, while lactate is the main intermediate in the conversion of starch to propionate. Lactic bacteria tolerate low pH conditions, and therefore are able to use H_2 and compete with methanogens even in

unfavorable pH conditions.

1.2.4. Inhibition of methane production in ruminants

Of the many alternative approaches to reducing methane, both in terms of reduction per animal and reduction per unit of animal product, the most promising areas are the development of new anti-methanogenic compounds or alternative electron acceptors in the rumen and the reduction of protozoa in the rumen.

Direct inhibition

Direct inhibition of methanogenesis by halogenated methane analogues and related compounds has been demonstrated both *in vitro* and *in vivo*. Chloroform reduces methanogenesis *in vitro* and *in vivo* (Clapperton, 1974), but is obviously not suitable for use in practice. Recently, 9, 10-anthroquinone has been shown to inhibit methanogenesis in mixed rumen microorganism populations *in vitro* (Garcia-Lopez et al., 1996; Kung et al., 1998) and to depress methane production in lambs over a 19-day period.

Ionophores

Ionophoric antibiotics such as monensin depress methane production in mixed rumen microbe populations *in vitro* (Van Nevel and Demeyer, 1992). This decrease is not due to a direct effect of the ionophores on methanogenic bacteria but rather due to a shift in the bacterial population from gram-positive to gram-negative organisms with a concurrent shift in fermentation from acetate to propionate.

Alternative growth promoters

Increasing awareness of antibiotic residues in animal products and the threat of bacterial antibiotic resistance in the wider environment has led to an increasing interest in alternatives to antibiotics as growth promoters. Martin (1998) suggested that dicarboxylic

organic acids such as malate may alter rumen fermentation in a manner similar to ionophores and that adding fumarate, a precursor of propionate, to rumen-simulating fermentors leads to an increase in propionate production with a stoichiometric decrease in methane production (Lopez et al., 1999b).

Stimulation of acetogens

An alternative strategy to reduce ruminal methanogenesis is to re-channel substrates for methane production into alternative products. Acetogenic bacteria in the hindgut of mammals and termites produce acetic acid by reducing CO_2 with H_2, and reductive acetogenesis acts as an important H_2 sink in hindgut fermentation (Demeyer and De Graeve, 1991). However, acetogens depress methane production when they are added to rumen fluid *in vitro*. Even if a stable population of acetogens cannot be established in the rumen, it might be possible to achieve the same metabolic activity using acetogens as a daily feed additive (Lopez et al., 1999a).

Methane oxidizers

Methane-oxidizing bacteria have been isolated from a wide range of environments, including the rumen, but studies with $^{13}CH_4$ tracers suggest that oxidation of methane to CO_2 is of little quantitative importance in the rumen (Valdes et al., 1996).

Defaunation

Methanogenic bacteria have been observed on the exterior surface of rumen ciliate protozoa (Vogels et al., 1980) and as endosymbionts within ciliates (Finlay et al., 1994). The inclusion of fat in ruminant diets depresses protozoa populations (Ikwuegbu and Sutton, 1982; Czerkawski et al., 1995), and at levels above 0.5% dry matter (DM) can significantly inhibit the breakdown of fiber in the rumen (Kowalcyzck et al., 1977; Machmuller and Kreuzer, 1997). However, the degree of effect varies according to the

type of fat used (Machmuller et al., 1998).

Probiotics

The most widely used microbial feed additives (live cells and growth media) are based on *Saccharomyces cerevisiae* (SC) and *Aspergillus oryzae* (AO). Their effects on rumen fermentation and animal productivity are wide ranging (see review by Martin and Nisbet, 1992). However, very little information is available on their effects on methane production.

Immunization

Shu et al. (1999) showed that immunization can successfully reduce the numbers of streptococci and lactobacilli in the rumen.

1.3. Objectives of this study

The aim of this dissertation was to develop a high-quality feed product for ruminants by utilizing self-sufficient feed including WCR and food by-products to prepare a TMR silage, and to determine the effects of fermented TMR on methane production in the rumen. The following objectives were established and experimentally tested via a series of eight different experiments (described in Chapters 2–5):

(1) To analyze the effects of food by-products on the fermentation quality of a WCR TMR silage, and its digestibility.

(2) To quantify the effects of fermented WCR TMR on ruminal fermentation and methane emission.

(3) To determine how fermented WCR TMR with a high LAC inhibits methane production.

Chapter 2

Effects of food by-products and lactic acid bacteria on the fermentation quality of TMR with WCR and its *in vitro* digestion characteristics

2.1. Effect of the addition of food by-products and lactic acid bacteria on fermentation quality of TMR silage with WCR silage and its *in vitro* dry matter digestibility, methane emission, and rumianl fermentation

2.1.1. Introduction

The technology necessary to prepare TMR with a balance of grains, proteins, minerals, and vitamins to meet the nutrient requirements of ruminants, and feed it to animals, has expanded in Japan. Though some studies have investigated the effects of food by-products on the fermentation quality of WCR TMR silage and its digestibility, but not related methane emissions in ruminants. Although WCR silage is typically of poor food quality compared to other forage crops such as corn and grass, because of low LAC due to a low soluble carbohydrate content (Yoshida et al., 1987; Enishi and Shijimaya, 1998; Cai, 2001), a lactic bacteria additive exclusively for WCR has been developed and is currently in use (Cai et al., 2003). In addition, adding fermented juice of epiphytic lactic acid bacteria (FJLB) improves the fermentation quality of WCR silage by increasing LAC (Hiraoka et al., 2003, 2006a,b). Accordingly, it is anticipated that if these two types of lactic bacteria are used together, they may further increase LAC and silage

quality. Moreover, because the conversion of lactic acid to propionate uses a great deal of H_2, such an increase in LAC could also help reduce methane production.

The objectives of the first study were to evaluate the effects of food by-products and lactic acid bacteria on fermentation quality and *in vitro* dry matter (DM) digestibility, ruminal volatile fatty acid (VFA), and methane production related to WCR TMR silage.

2.1.2. Materials and Methods

2.1.2.1. Preparation of WCR silage and FJLB

WCR (Haenuki) was cultivated using conventional methods in a paddy field on an experimental farm at Yamagata University, Japan. WCR was harvested at the full-ripe stage, prepared into a mini roll bale silage (50 kg), and stored outdoors (9–32°C) for 240 days. After fermentation, the moisture content was 65.8%, pH was 5.12, and the FM lactic acid and acetic acid concentrations were 0.55% and 0.09%, respectively.

FJLB was prepared according to the methods described by Takahashi et al. (2005). Briefly, 200 g of cut WCR silage were macerated in 1 L of water by adding 2% (weight/volume) sucrose, and preserved anaerobically at 30°C for 2 days. The macerate was then filtered through double cheesecloth and blended with 2% (w/v) sucrose.

2.1.2.2. Preparation of WCR TMR silage

WCR silage was cut to a length of 2 cm. As shown in Table 2.1.1, TMR was prepared using compound feed (Kitanihon-kumiai Feed, Yamagata, Japan); WCR silage; dried beet pulp (Zennou, Tokyo); a vitamin-mineral supplement (Snow Brand Seed, Iwate, Japan; vitamin A, 5,000,000 IU/kg; vitamin D3, 1,000,000 IU/kg; vitamin E, 2 g/kg; vitamin K3, 0.2 g/kg; vitamin B1, 0.5 g/kg; vitamin B2, 1 g/kg; vitamin B6, 0.1 g/kg; vitamin B12, 0.001 g/kg; nicotinic acid, 6 g/kg; choline chloride, 2 g/kg; calcium

pantothenate-D, 10 g/kg; Mn, 0.16 g/kg; Zn, 0.7 g/kg; Fe, 0.55 g/kg; Cu, 0.14 g/kg; I, 0.33 g/kg; Co, 0.04 g/kg; methionine, 1 g/kg; lidocaine hydrochloride, 0.5 g/kg); a food byproduct, either dry TC (Zennou, Tokyo, Japan; moisture, 8.7%), RB (Yamagata University Farm, Yamagata, Japan), or WGTW (Marubishi Food, Yamagata, Japan; moisture, 82.8%); and lactic bacteria, both *Lactobacillus plantarum Chikuso-1* (Snow Brand Seed, Sapporo, Japan) and FJLB. The ingredients and proportions used are listed in Table 2.1.2. In each silage, the proportions of WCR silage, feed concentrate, dried beet pulp, and vitamin–mineral supplement were fixed at 30%, 25%, 13.5%, and 1.5% of TMR DM, respectively, while the remaining 30% of TMR DM consisted of a mix of TC, RB, and WGTW. Furthermore, experimental treatments included either an untreated control group (no lactic bacteria added) or treated group (i.e., LAB; lactic bacteria added). The LAB group included the addition of both *Lactobacillus plantarum Chikuso-1* at a rate of 5 mg/kg (5 ppm) of fresh TMR, and FJLB at a rate of 2% (v/w) of fresh TMR. TMR moisture was adjusted with water to 65%. Silages were prepared using a small-scale system of silage fermentation. Approximately 1 kg TMR was packed into plastic film bags (Hiryu BN-12 type, 270 mm × 400 mm; Asahikasei, Tokyo, Japan), and the bags were sealed with a vacuum sealer (SQ303; Sharp, Osaka, Japan). Three silos per treatment were prepared and stored in a room (20–25°C) for 60 days. The contents of crude protein (CP) and TDN for WCR silage, beet pulp, TC, RB, and WGTW were calculated based on the Standard Tables of Feed Composition in Japan (2001) and Xu et al. (2003).

2.1.2.3. Chemical and microbial analyses

The WCR silage, WGTW, and TMR silages were dried in a forced draft oven at 60°C for 48 h, ground, and filtered through a 2-mm mesh screen with a sample mill (Foss

Tecator; Akutalstuku, Tokyo, Japan). The content of moisture, ash, nitrogen, ether extract, and crude fiber of WCR, feed concentrate, beet pulp, TC, RB, and WGTW were determined according to conventional methods (Horii et al., 1971). Analyses of the neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of WCR, feed concentrate, beet pulp, TC, RB, and WGTW were conducted according to Van Soest et al. (1991). Heat-stable amylase and sodium sulfite were used in the NDF procedure, and the results were expressed without residual ash. The fermentation products of the WCR and TMR silages were determined from cold-water extracts. Wet silage (50 g) was homogenized with 200 ml of sterilized distilled water and stored at 4°C overnight (Cai et al., 1999). The pH of both silages was determined using a glass electrode pH meter (D-21; Horiba, Kyoto, Japan), and LAC was analyzed following Barker and Summerson (1941). Volatile basic nitrogen (VBN) was determined as described by Conway (1962). Silage and ruminal fluids were steam-distilled and titrated using sodium hydroxide to measure total VFA (Hamada, 1971). Dried VFA salt was separated and quantified using gas chromatography (G-5000A; Hitachi, Tokyo, Japan) equipped with a thermal conductivity detector and a glass column (Unisole F-200, 3.2 mm × 2.1 m). The analytical conditions were as follows: column oven temperature, 140°C; injector temperature, 210°C; detector temperature, 250°C. To assess silage quality, Flieg's scores were determined based on LAC and VFA, and V-scores were determined based on NH_3-N/total N and VFA concentrations (Japanese Grassland Agriculture and Forage Seed Association, 2001).

2.1.2.4. Cultures and incubations

TMR silage, ground into 0.5-mm powder, was used as substrate for *in vitro* culture.

Two adult sheep (average initial body weight, 78.5 kg) fitted with rumen cannulae were used as donors of ruminal fluid. Wethers were fed basal diets of 50% reed canary

grass (*Phalaris arundinacea* L.) hay and 50% commercial feed concentrate (Koushi-Ikusei-Special; Kitanihon-Kumiai-Feed, Miyagi, Japan) at maintenance energy level (20 g DM/kg body weight daily) and had free access to clean drinking water. Wethers were fed once daily at 09:00 h and were cared for according to the animal care and use guidelines of the Faculty of Agriculture, Yamagata University.

Rumen fluid was collected through the rumen cannulae 2 h after feeding, diverted to plastic bottles. The fluids were then filtered through four layers of cheesecloth and combined on an equal volume basis. The compound filtrate was mixed with CO_2-bubbled McDougal's artificial saliva (pH 6.8; McDougal, 1948) at a ratio of 1:4 (v/v). Then 50 ml diluted rumen fluid were transferred to 128-ml serum bottles containing 0.5 g of ground TMR, and flushed with O_2-free CO_2. The tubes were capped with a butyl rubber stopper and sealed with an aluminum cap. Incubation was done in triplicate at 39°C for 6 h in a water bath with a reciprocal shaker (100 strokes/min).

2.1.2.5. Analysis of fermentation products

To terminate fermentation at the end of incubation, 25 μL of formaldehyde solution (35%) were injected into serum bottles, which were immediately sealed and cooled at room temperature. The gas sample was collected using an air syringe from the serum bottles and injected into a gas chromatograph (GC323; GL Sciences, Tokyo, Japan) equipped with a thermal conductivity detector and stainless used steel column (WG-100 SUS 1.8 m × 6.35 mm OD (outer diameter)), and then methane production from each serum bottle was measured. The analytical conditions were as follows: column oven temperature, 50°C; injector temperature, 50°C; detector temperature, 50°C.

2.1.2.6. In vitro dry matter digestibility, and the production of methane and VFA

Separate sub-samples of the supernatant were taken to determine pH and VFA

concentration. The bottles were rinsed with warm water to remove all solid residues. The residues were oven-dried at 60°C and stored for further analysis. Then 2 g of dried residue were oven-dried at 135°C and stored to determine DM digestibility (Horii et al., 1971).

2.1.2.7. Statistical analyses

Analyses were carried out using the general linear model procedure (SAS institute, Cary, NC, USA). Data on fermentative characteristics, *in vitro* DM digestibility, and ruminal methane and VFA production were subjected to a two-way analysis of variance (ANOVA) with LAB and food by-products as the two factors. Tukey's test was used to identify differences ($P < 0.05$) between means.

2.1.3. Results

2.1.3.1. Fermentation characteristics of WCR TMR silage

The fermentation characteristics of the WCR TMR silages are shown in Table 2.1.3. There were notable differences ($P < 0.05$) in pH, LAC, FM acetic acid levels, and V-scores among LAB and food byproduct treatments. Furthermore, there were notable interactive effects ($P < 0.05$) between LAB treatments on pH and V-score.

The pH of the WCR silage was 5.12 and those of all TMR silages with food by-products were under 3.8. Treating TMR silage with LAB further reduced the mean pH ($P < 0.05$). The LAC of the WCR silage was 0.5% and those of TMR silages with food by-products were 2.72–3.41% and 2.92–3.68% for control and LAB treatments, respectively. LAB treatments had slightly higher LACs than controls (0.34%; $P < 0.05$). Acetic acid levels were 0.18–0.28% and 0.15–0.24% for LAB and control treatments, respectively, and the mean acetic acid level for LAB treatments was 0.20% lower ($P < 0.05$) than for control treatments. In control treatments, acetic acid levels varied as

follows according to the food byproduct added (i.e., 30% WGTW, 30% TC, or 30% RB): 0.28%, 0.24%, and 0.20% for WGTW, TC, and RB, respectively. Although propionic acid and butyric acid were detected, both acids were at negligible levels. VBN concentrations were 3.18–4.39% and 3.16–4.26% for control and LAB treatments, respectively, and did not differ significantly. TMR silage with 30% WGTW had the lowest levels ($P < 0.05$) among the silages with or without LAB. Furthermore, the Flieg's scores for all silages in both treatments were 100 or higher, and the V-scores of silages with and without LAB were 99.7–100 and 99.3–100, respectively.

2.1.3.2. DM digestibility and the production of methane and VFA of WCR TMR silage

DM digestibility, methane production, and VFA concentrations *in vitro* after 6 h of incubation of WCR TMR silages are shown in Table 2.1.4. There were notable differences ($P < 0.05$) in all variables for silages with added food by-products, and in DM digestibility, methane, and VFAs such as acetic acid, propionic acid, isovaleric acid, and the A/P ratio between silages with and without LAB. There were also significant interactive effects ($P < 0.05$) on acetic acid and valeric acid between LAB and food by-products for pH and V-score.

DM digestibility for silages with and without LAB was 33.9–43.6% and 29.2–42.4%, respectively, and was significantly higher for silages with LAB ($P < 0.05$). Silages with 30% RB were the highest among LAB and control groups, and were significantly higher ($P < 0.05$) than those with 10% RB and 20% WGTW. Methane production in silages with and without LAB were 10.1–14.6 L/kg digestible DM (DDM) and 11.8–16.4 L/kg DDM, respectively. The molar proportions of acetic acid in silages with LAB were lower ($P < 0.05$) than in silages without LAB, but did not differ among silages without LAB. Propionic acid was higher in LAB silages ($P < 0.05$) than no-LAB silages; LAB silages

with TC + RB had higher levels ($P < 0.05$) than no-LAB silages with 30% WGTW or TC + WGTW. Butyric acid in silages with and without LAB was 13.97–23.22 mol% and 14.58–24.44 mol%, respectively, a non-significant difference. However, among LAB silages, those with 30% TC or with TC + RB had lower levels ($P < 0.05$) than those with 30% WGTW or with TC + WGTW. Isovaleric acid in silages with and without LAB were 0.23–0.71 mol% and 0.34–1.04 mol%, respectively, i.e., significantly lower in LAB silages ($P < 0.05$). Valeric acid did not differ between LAB and no-LAB silages. The A/P was lower ($P < 0.05$) in LAB than in no-LAB silages, but did not differ among no-LAB silages. Among LAB silages, those with 10% TC and 20% RB had a lower A/P ($P < 0.05$) than those with either 20% or 30% WGTW. Total VFA did not differ between silages with and without LAB, but did vary according to the combination of food by-products used: silages with 30% TC had higher levels ($P < 0.05$) than silages with 30% RB or 30% WGTW, with or without LAB.

2.1.4. Discussion

In recent years, advances in the TMR feeding system in the dairy industry have led to increasing interest in local, low-cost feed resources, including various food by-products (Seki et al., 2000; Goga et al., 2001). Although some studies have focused on WCR preparation (Matsuyama et al., 2005, 2006) and the fermentation quality and nutritive value of WCR silage, no previous reports have examined how adding food by-products affects the fermentation quality, LAC, DM digestibility, and ruminal methane and VFA production of WCR TMR silage. Therefore, the present study considered these characteristics using food by-products such as TC, RB, and WGTW with or without LAB (i.e., *Lactobacillus plantarum Chikuso-1*, FJLB). Fermented TMR

silage prepared using assorted feed loses some nutrition during the fermentation process but has a higher fermentation quality; it can be preserved for a long period of time, ruminants prefer it over fresh TMR silage, and its deterioration and lipid oxidation can be controlled. That is, its utility as a feed is high, especially in the summer (Shioya et al., 2007). Using food by-products not only reduces feed costs but also improves feed self-sufficiency in Japan. Because *Chikuso-1* and FJLB both improve silage fermentation (Cai et al., 2003; Hiraoka et al., 2003; Takahashi et al., 2005), using the two types of LAB jointly to prepare silage further promotes lactic acid fermentation and silage quality.

The present experiment clearly demonstrated that both LAB and food by-products influenced the fermentation quality of silage. All TMR silages were well prepared, had a low pH, high LAC, low NH_3-N/total nitrogen (TN), and high Flieg's scores or V-scores. Furthermore, adding LAB led to even lower pH and higher LAC in TMR silages. This is because of the high nonfibrous carbohydrate (NFC) content of both feed concentrate and beet pulp, which supply the necessary carbohydrates for LAB fermentation, ensuring that lactic acid fermentation can progress with the addition of more LAB (Cai et al., 2003; Hiraoka et al., 2006b). Among all silages, those with 30% TC had the highest LAC. This is because TC has 16% NFC, much more than WGTW (9.5%); although RB has 16.7% NFC, slightly more than TC, it also has a high level of oxidation lipid, which hinders lactic acid fermentation (Yokota and Ohshima, 1997). Moisture content may also affect the fermentation quality of silage. In the present experiment, moisture content was adjusted to 65%, according to Xu et al. (2004b). This resulted in high-quality silage, and thus 65% moisture is suitable for lactic bacteria fermentation.

Seki et al. (2000) reported that 15% food byproduct per unit of DM was suitable for preparing TMR. In the present study, although food by-products were combined at 30%

TMR (DM basis) for preparing silages, the contents of CP and TDN in silages were 12.8–17.0% and 68.2–75.1%, respectively. According to the Japanese Feeding Standard for Beef Cattle (Agriculture, Forestry, and Fisheries Research Council Secretariat, MAFF, 2000), the required levels of CP and TDN for the fattening prophase of beef cattle are 12.9–15.7% and 68–75%, respectively, and thus the TMR silages prepared in this experiment were practical.

The *in vitro* experiment clearly showed that DM digestibility, methane production, and the molar proportion of acetic acid and propionic acid were influenced by both LAB and food by-products, and that the A/P ratios in silages with LAB were lower than in silages without LAB. That is, DM digestibility was higher in LAB silages than no-LAB silages. The presence of relatively large populations of LAB may have reduced the active period of other microorganisms in the initial fermentation stage (Cai and Ohmomo, 1995), leading to this result.

Because RB is a high-energy feed that mainly contains CP and ether extract (EE) and has high ruminal digestibility (Enishi and Kawashima, 2003), TMR silages with 30% RB also had high *in vitro* digestibility.

In the rumen, the production of both propionic acid and methane uses H_2, and thus propionic acid formation can be considered a competitive pathway for H_2 use (Leng, 1970; Moss et al., 2000). Therefore, silages with a high LAC use a large amount of H_2 to produce propionic acid, thereby decreasing methane production. In the present experiment, although total VFA did not differ between silages with and without LAB, mean acetic acid levels were lower in LAB silages than no-LAB silages, while mean propionic acid levels were higher in LAB silages. A previous study found that as the NFC content of feed decreases, total VFA content also decreases but the A/P ratio increases

(Batajoo and shave, 1994). The present experiment is in agreement with these previous findings: WGTW silage had less NFC than either TC or RB silage, and a lower total VFA but a higher A/P ratio.

Adding fat to feed may inhibit methanogen activity in the rumen, which in turn reduces methane production, although the effect depends at least partly on the fat source used (Dong et al., 1997; Machmuller et al., 1998). However, the effects of fat on methane production are not limited to those mediated via rumen protozoa, and lipids inhibit methanogensis even in the absence of rumen protozoa (Broudiscou et al., 1990; Dohme et al., 1999), possibly due to the toxicity of long chain fatty acids to methanogenic bacteria (Prins et al., 1972; Henderson, 1973). In addition, previous studies have reported decreased methane production by ruminants fed feed with safflower oil (Horiguchi et al., 2002), sunflower oil (McGinn et al., 2004), and coconut oil (Jordan et al., 2006). Free lauric acid and some lauric acid-rich oils may improve rumen fermentation of high-grain diets (Yabuuchi et al., 2006). In the present experiment, ruminal methane production per unit DDM tended to decrease in silages with or without LAB in the following order: silages with 30% WGTW (15.8 and 14.6 L/kg DDM), 30% TC (12.8 and 12.8 L/kg DDM), and 30% RB (11.8 and 10.1 L/kg DDM). There was a notable difference between silages with 30% WGTW and those with 30% RB, likely because of the higher EE and unsaturated fatty acid levels in RB (Yokota and Ohshima, 1997), each of which may depress methanogen activity and therefore methane production. Similar to RB, there are large amounts of unsaturated fatty acids such as oleic, linoleic, and linolenic acids in TC, which is made of soybean (Kagawa, 2005). However, methane production did not differ between silages with 30% TC and 30% WGTW, likely due to the lower levels of polyunsaturated fatty acid (PUFA) in TC compared to RB (Kagawa, 2005).

Using food by-products (i.e., TC, RB, and WGTW) clearly improved the quality of WCR TMR silage. Adding 30% TC increased LAC; adding 30% RB increased DM digestibility and decreased methane production; and adding LAB promoted lactic acid fermentation and DM digestibility and reduced methane production. Future studies should examine the moderating effect of WCR TMR silage with high LAC on ruminant methane production using feeding experiments (*in vivo*).

2.1.5. Summary

TMR silages were prepared using tofu cake (TC), rice bran (RB), and wet green tea waste (WGTW) at 10, 20, and 30% DM, respectively, with WCR silage and commercial formula feed. Twenty experimental groups were prepared with or without lactic acid bacteria (*Chikuso-1*, 5 ppm FM, and fermented juice of epiphytic lactic acid bacteria [FJLB], 2% FM). All experimental silages were good quality because pH values were less than 3.8, and lactic acid content was more than 2.72% (FM). The LAC for 30% TC TMR silage was highest among all treatments. The *in vitro* DM digestibility for 30% RB TMR silage was highest among all treatments, while methane production per DDM for 30% RB silage was lowest among treatments. In addition, silages supplemented with lactic acid bacteria had lower pH ($P < 0.05$), higher LAC ($P < 0.05$), higher *in vitro* DM digestibility ($P < 0.05$), and lower methane production per DDM ($P < 0.05$) than controls.

Table 2.1.1. Chemical composition of WCRS, concertrate, beet pulp, tofu cake, rice bran and green tea grounds used in TMR[1] silages

	WCRS[11]	Concentrate[12]	Beet pulp	DTC[13]	RB[14]	WGTW[15]
Moisture (%)	65.8	11.8	9.3	8.7	10.6	82.8
CP[2] (% DM[3])	5.7	16.7	8.4	30.1	16.9	32.0
EE [4] (% DM)	2.3	3.8	0.7	12.2	24.5	5.4
NFE[5] (% DM)	48.5	69.8	68.5	40.2	37.5	40.5
CF[6] (% DM)	28.1	4.7	17.3	13.2	8.7	18.8
NFC[7] (% DM)	23.1	60.0	33.7	15.8	16.8	9.5
CA [8] (% DM)	15.4	5.1	5.1	4.3	12.4	3.3
ADF[9] (% DM)	33.6	8.7	25.6	22.2	12.8	32.1
NDF[10] (% DM)	53.5	14.4	52.1	37.7	29.5	49.8

[1]Total mixed ration.

[2]Crude protein.

[3]Dry matter.

[4]Ether extract.

[5]Nitrogen free extract.

[6]Crude fiber.

[7]Non-fibrous carbohydrate(100 – CP – EE – NDF – CA).

[8]Crude ash.

[9]Acid detergent fiber.

[10]Neutral detergent fiber.

[11]Whole crop rice silage.

[12]Formula feed ("Koushi Ikusei Special Mash" made by Zenno, TDN: 70.0%, CP:12.0% in fresh matter).

[13]Dry tofu cake.

[14]Rice bran.

[15]Wet green tea waste.

Table 2.1.2. Ingregient and nutrient composition of TMR[1] silage

	Mixing ration of materials									
Ingredient										
WCRS[2] (% DM[3])	30	30	30	30	30	30	30	30	30	30
Concentrate[4] (% DM)	25	25	25	25	25	25	25	25	25	25
Vitamin supplement[5] (% DM)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Beet pulp (% DM)	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5
DTC[6] (% DM)	10	30	20	10	0	0	0	0	10	20
RB[7] (% DM)	10	0	10	20	30	20	10	0	0	0
WGTW[8] (% DM)	10	0	0	0	0	10	20	30	20	10
Nutrient composition										
CP[9] (% DM)	15.4	16.4	15.2	14.0	12.8	14.2	15.6	17.0	16.8	16.6
TDN[10] (% DM)	72.5	75.1	74.8	74.6	74.3	72.3	70.2	68.2	70.5	72.8

[1]Total mixed ration.

[2]Whole crop rice silages.

[3]Dry matter.

[4]Formula feed ("Koushi Ikusei Special Mash" made by Zenno, TDN: 70.0%, CP:12.0% in fresh matter).

[5]Commercial vitamin-mineral supplement product (Snow brand seed, Iwate, Japan).

[6]Dry tofu cake.

[7]Rice bran.

[8]Wet green tea waste.

[9]Crud protein.

[10]Total digestible nutrents.

Table 2.1.3. Fermentative characteristics of TMR[1] silages

	Moisture (%)	pH	Lactic acid	Acetic acid	Propionic acid	Butyric acid	NH_3-N /TN[5] (%)	Frieg's mark	V-score
			(%FM[4])						
Control									
DTC:RB:WGTW[2]									
10:10:10	64.7	3.66^{b}	2.94^{ab}	0.23^{cd}	0.01	0.01	4.17^{ab}	100	99.7^{b}
30:0:0	63.8	3.78^{a}	3.41^{a}	0.24^{bd}	0.00	0.01	4.30^{ab}	100	99.7^{b}
20:10:0	62.4	3.67^{b}	3.23^{ab}	0.20^{f}	0.00	0.01	4.32^{ab}	100	100^{a}
10:20:0	63.0	3.69^{b}	3.00^{ab}	0.18^{f}	0.00	0.00	4.39^{a}	100	100^{a}
0:30:0	62.3	3.66^{b}	2.99^{ab}	0.20^{ef}	0.00	0.00	4.12^{ab}	100	100^{a}
0:20:10	63.7	3.65^{b}	2.93^{ab}	0.22^{de}	0.00	0.00	4.11^{ab}	100	99.8^{ab}
0:10:20	64.4	3.65^{b}	3.05^{ab}	0.25^{b}	0.01	0.00	3.84^{ab}	100	99.5^{c}
0:0:30	63.8	3.68^{b}	2.72^{b}	0.28^{a}	0.01	0.00	3.18^{c}	100	99.3^{d}
10:0:20	63.4	3.69^{b}	2.99^{ab}	0.25^{b}	0.01	0.01	3.75^{bc}	100	99.5^{c}
20:0:10	62.5	3.70^{b}	3.18^{ab}	0.25^{b}	0.01	0.01	3.93^{ab}	100	99.5^{c}
Mean	63.4	3.68	3.04	0.23	0.00	0.01	4.01	100	99.7
LAB ("*Chikuso-1* "+FJLB)									
DTC:RB:WGTW									
10:10:10	64.5	3.65^{abc}	3.36^{ab}	0.19^{abc}	0.00	0.01	4.15^{ab}	100	100^{a}
30:0:0	63.6	3.66^{ab}	3.68^{a}	0.19^{abc}	0.00	0.00	4.20^{ab}	100	100^{a}
20:10:0	63.9	3.63^{abcd}	3.62^{a}	0.18^{bc}	0.00	0.00	4.23^{a}	100	100^{a}
10:20:0	63.8	3.65^{abc}	3.55^{ab}	0.15^{c}	0.00	0.00	4.26^{a}	100	100^{a}
0:30:0	64.1	3.64^{abcd}	3.27^{ab}	0.16^{c}	0.00	0.01	4.12^{ab}	100	100^{a}
0:20:10	63.6	3.59^{d}	3.34^{ab}	0.20^{abc}	0.00	0.01	3.86^{ab}	100	100^{a}
0:10:20	64.3	3.60^{cd}	3.36^{ab}	0.24^{a}	0.00	0.01	3.78^{ab}	100	99.7^{b}
0:0:30	64.1	3.60^{bcd}	2.92^{b}	0.24^{a}	0.00	0.00	3.16^{c}	100	99.7^{b}
10:0:20	63.9	3.64^{abcd}	3.11^{ab}	0.22^{ab}	0.01	0.01	3.68^{bc}	100	99.8^{ab}
20:0:10	63.6	3.67^{a}	3.56^{ab}	0.22^{ab}	0.01	0.01	3.88^{ab}	100	99.8^{ab}
Mean	63.9	3.63	3.38	0.20	0.00	0.00	3.93	100	99.9
SEM[3]	0.38	0.01	0.13	0.01	0.00	0.00	0.10	0.00	0.03
Statistical significance									
LAB	0.0082	<0.0001	<0.0001	<0.0001	NS[6]	NS	0.1359	NS	<0.0001
Food by-products	0.1118	<0.0001	0.0003	<0.0001	NS	NS	<0.0001	NS	<0.0001
Interractions	0.2185	0.0110	0.9170	0.3522	NS	NS	0.9926	NS	0.0002

[1]Total mixed ration.

[2]Dry tofu cake : Rice bran : Wet green tea waste.

[3]Standard error of means.

[4]Fresh matter.

[5]Total nitrogen.

[6]not significant.

[a,b,c,d,e,f]Means in the same column with different superscripts differ significantly ($P < 0.05$).

Table 2.1.4. Measurements of dry matter digestibility, methane production and VFA concentration *in vitro* after 6 hours incubation of TMR[1] silages

	DM[4] digestibility (%)	Methane production (L/kg DDM[5])	Acetic acid	Propionic acid	Butyric acid	Iso-valeric acid	Valeric acid	A:P[6]	Total VFA (mmol/dl)
					(mol%)				
Control									
DTC:RB:WGTW[2]									
10:10:10	33.0^{ab}	15.3^{ab}	41.86	35.95^{bc}	17.80^{bc}	1.04^{a}	3.34	1.16	6.07^{bcd}
30:0:0	36.1^{ab}	12.8^{bc}	44.16	37.50^{ab}	14.73^{c}	0.76^{ab}	2.85	1.18	6.79^{a}
20:10:0	33.3^{ab}	15.1^{ab}	43.57	38.33^{a}	14.86^{c}	0.68^{ab}	2.56	1.14	6.43^{abc}
10:20:0	35.8^{ab}	13.3^{ac}	43.20	39.45^{a}	14.58^{c}	0.49^{ab}	2.28	1.10	6.53^{ab}
0:30:0	42.4^{a}	11.8^{c}	40.22	37.54^{ab}	18.40^{bc}	0.60^{ab}	3.24	1.07	6.09^{bcd}
0:20:10	37.1^{ab}	14.1^{ac}	41.74	34.65^{cd}	19.56^{abc}	0.51^{ab}	3.54	1.21	5.79^{cd}
0:10:20	29.2^{b}	16.4^{a}	38.88	33.36^{d}	24.44^{a}	0.59^{ab}	2.73	1.17	5.53^{d}
0:0:30	33.6^{ab}	15.8^{ab}	38.41	33.97^{cd}	22.94^{ab}	0.52^{ab}	4.17	1.13	5.89^{bcd}
10:0:20	34.9^{ab}	15.9^{ab}	41.09	34.86^{cd}	20.77^{ab}	0.34^{b}	2.94	1.18	5.96^{bcd}
20:0:10	38.4^{ab}	14.5^{ac}	40.18	34.94^{cd}	20.90^{ab}	0.39^{ab}	3.59	1.15	6.21^{abcd}
Mean	35.4	14.5	41.33	36.06	18.90	0.59	3.12	1.15	6.13
LAB ("*Chikuso-1* "+FJLB)									
DTC:RB:WGTW									
10:10:10	35.8^{ab}	13.4^{ab}	40.02^{ab}	35.58^{c}	19.76^{abc}	0.71^{a}	3.93^{ab}	1.13^{ab}	6.00^{bc}
30:0:0	36.4^{ab}	12.8^{ab}	40.51^{ab}	39.44^{ab}	16.25^{bc}	0.59^{ab}	3.20^{ab}	1.03^{ab}	6.41^{ab}
20:10:0	37.7^{ab}	12.4^{ab}	44.02^{a}	39.17^{ab}	13.97^{c}	0.43^{ab}	2.40^{ab}	1.13^{ab}	6.28^{abc}
10:20:0	36.8^{ab}	12.2^{ab}	38.47^{ab}	40.41^{a}	17.41^{abc}	0.35^{ab}	3.35^{ab}	0.95^{b}	6.64^{a}
0:30:0	43.6^{a}	10.1^{b}	36.70^{b}	36.70^{bc}	21.57^{ab}	0.60^{ab}	4.43^{ab}	1.00^{ab}	5.82^{c}
0:20:10	37.4^{ab}	13.5^{ab}	36.19^{b}	35.47^{c}	23.22^{a}	0.56^{ab}	4.56^{a}	1.02^{ab}	6.06^{abc}
0:10:20	33.9^{b}	13.7^{ab}	40.11^{ab}	34.18^{c}	21.84^{ab}	0.55^{ab}	3.32^{ab}	1.17^{a}	5.80^{c}
0:0:30	36.6^{ab}	14.6^{a}	41.05^{ab}	34.69^{c}	21.48^{ab}	0.40^{ab}	2.39^{ab}	1.18^{a}	5.75^{c}
10:0:20	36.5^{ab}	14.2^{a}	42.25^{ab}	34.83^{c}	19.50^{abc}	0.38^{ab}	3.03^{ab}	1.21^{a}	5.79^{c}
20:0:10	40.8^{ab}	13.0^{ab}	40.91^{ab}	35.35^{c}	21.16^{ab}	0.23^{b}	2.35^{b}	1.16^{ab}	5.90^{bc}
Mean	37.6	13.0	40.02	36.58	19.62	0.48	3.30	1.10	6.04
SEM[3]	1.57	0.56	1.18	0.42	1.07	0.09	0.41	0.04	0.11
Statistical significance									
LAB	0.0157	<0.0001	0.0349	0.0461	0.2058	0.0273	0.418	0.014	0.1382
Food by-products	<0.0001	<0.0001	0.0088	<0.0001	<0.0001	0.0004	0.049	0.004	<0.0001
Interractions	0.9549	0.6834	0.031	0.5083	0.1625	0.7553	0.043	0.074	0.1329

[1]Total mixed ration.

[2]Dry tofu cake : Rice bran : Wet green tea waste.

[3]Standard error of means.

[4]Dry matter.

[5]Digestible dry matter.

[6]Acitic acid:Propionic acid.

[a,b,c,d]Means in the same column with different superscripts differ significantly ($P < 0.05$).

2.2. Effect of adding lactic acid bacteria and molasses on fermentation quality of TMR with WCR, and its *in vitro* dry matter digestibility, methane emission, and ruminal fermentation

2.2.1. Introduction

There is a growing body of research in Japan on the utilization of WCR as a feed for ruminants (Nishino et al., 2007). This is due in part to the fact that 400,000 ha of paddy fields are currently unused, while about a quarter of the domestic roughage demand is supplied by imports, mainly from China, Australia, and North America (Shioya and Cai, 2004). The paddy fields could serve as manure deposition sites, thereby recycling local bio-resources and enhancing crop farming and animal husbandry. A number of WCR cultivars have been bred exclusively as feed for ruminants. The average content of crude protein and metabolizable energy of one such cultivar was 60–70 g/kg (DM) and 8.3 MJ/kg (DM), respectively, equivalent to hay and silage prepared from late-harvested grasses (Shioya and Cai, 2004). The crop is harvested (typically as round-bale silage) around the yellow-ripe stage to maximize the yield of total digestible nutrients. WCR is usually insufficient in sugars and lactic acid bacteria (LAB; Cai et al., 2003), and may produce silages rich in ethanol rather than lactic acids and VFA (Yamamoto et al., 2004). This could be attributed to the structure of the rice plant; the hollow stem may increase the air in a silo, facilitating yeast growth especially in the early ensiling period (Shioya and Cai, 2004). The preservation of forage crops as silage depends on the production of sufficient acid to inhibit the activity of undesirable microorganisms under anaerobic conditions. LAB present in forage crops convert sugar into lactic acid during the ensiling

process. This reduces the pH and preserves the forage. However, there is so little sugar and LAB in forage crops such as WCR that very little lactic acid is produced (Cai et al., 2003) and the pH does not become low enough to preserve silage. *Lactobacillus plantarum Chikuso-1* has great potential as an inoculant for paddy rice forage (Cai et al., 1999, 2003; Cai, 2001).

Furthermore, the use of fermentation aids to increase the rate of fermentation of ensiled forage crops has been the subject of many investigations (e.g., Alli et al., 1984). Molasses has been used extensively as a fermentation aid, as it provides fermentable sugars for the production of organic acids (Alli et al., 1984), and silage prepared with molasses has a lower pH, higher residual water-soluble carbohydrate levels, greater quantities of lactic acid, lower levels of volatile nitrogen, decreased dry DM loss, and reduced levels of volatile nitrogen compared to silage without molasses. Weinberg et al. (2003) also reported a high LAC in silage ensiled with straw and molasses. Moreover, experiment 1 (described above) of the present study showed that WCR TMR silage with 30% TC had the highest LAC among all silages with food by-products. Therefore, adding LAB, molasses, and TC should produce a WCR TMR silage with an even higher LAC.

The purpose of this experiment was to study the effects of LAB and molasses on the fermentation quality of TMR with WCR and TC, and its *in vitro* DM digestibility, methane emission, and ruminal fermentation.

2.2.2. Materials and Methods

2.2.2.1. Silage preparation

WCR (Haenuki) was cultivated using conventional methods in a paddy field on an experimental farm at Yamagata University, Japan, harvested at the full-ripe stage, and cut

to a length of 2 cm. As shown in Table 2.2.1, TMR was prepared using compound feed (Kitanihon-Kumiai Feed, Yamagata, Japan); WCR; dried beet pulp; a vitamin–mineral supplement (Snow Brand Seed, Iwate, Japan; vitamin A, 5,000,000 IU/kg; vitamin D3, 1,000,000 IU/kg; vitamin E, 2 g/kg; vitamin K3, 0.2 g/kg; vitamin B1, 0.5 g/kg; vitamin B2, 1 g/kg; vitamin B6, 0.1 g/kg; vitamin B12, 0.001 g/kg; nicotinic acid, 6 g/kg; choline chloride, 2 g/kg; calcium pantothenate-D, 10 g/kg; Mn, 0.16 g/kg; Zn, 0.7 g/kg; Fe, 0.55 g/kg; Cu, 0.14 g/kg; I, 0.33 g/kg; Co, 0.04 g/kg; methionine, 1 g/kg; lidocaine hydrochloride, 0.5 g/kg); TC food byproduct (Zenno, Tsuruoka, Japan); molasses (sugarcane; Dai-Nippon Meiji Sugar Co., Tokyo, Japan), and LAB (*Lactobacillus plantarum Chikuso-1*; Snow Brand Seed, Sapporo, Japan). The TMR ingredients and proportions are shown in Table 2.2.2. Treatments included a control silage (i.e., neither molasses nor LAB added) or a silage with molasses and LAB (M-LAB) addition, and the addition of TC at 30% DM. Moisture was adjusted with water to approximately 65%. Silages were prepared using a small-scale system of silage fermentation. Approximately 1 kg TMR was packed into plastic film bags (Hiryu BN-12 type, 270 mm × 400 mm; Asahikasei, Tokyo, Japan), and the bags were sealed with a vacuum sealer (SQ303; Sharp, Osaka, Japan). A total of three silos per treatment were prepared and stored in a room (20–25°C) for 60 days. The content of CP and TDN for WCR silage, beet pulp, and TC were calculated according to the Standard Tables of Feed Composition in Japan (2001).

2.2.2.2. Chemical and microbial analyses

The TMR silages were dried in a forced draft oven at 60°C for 48 h and ground into a 2-mm powder with a sample mill (Foss Tecator; Akutalstuku, Tokyo, Japan). Moisture, ash, nitrogen, EE, and crude fiber (CF) content of WCR, feed concentrate, beet pulp, and TC were determined using conventional methods (Horii et al., 1971). Analysis of the

neutral detergent fiber (NDF) and acid detergent fiber (ADF) content of WCR, feed concentrate, beet pulp, TC, RB, and WGTW were made following Van Soest et al. (1991). Heat-stable amylase and sodium sulfite were used in the NDF procedure, and the results were expressed without residual ash. The fermentation products of the WCR and TMR silages were determined using cold-water extracts. Wet silage (50 g) was homogenized with 200 ml sterilized distilled water and stored at 4°C overnight (Cai et al., 1999). The pH of the WCT and TMR silage was determined using a glass electrode pH meter (D-21; Horiba, Kyoto, Japan). Lactic acid was analyzed using the methods of Barker and Summerson (1941). Volatile basic nitrogen (VBN) was determined as described by Conway (1962). To measure total VFA, silage and ruminal fluid were steam-distilled and titrated using sodium hydroxide (Hamada, 1971). Dried VFA salt was separated and quantified using gas chromatography (G-5000A; Hitachi, Tokyo, Japan) equipped with a thermal conductivity detector and a glass column (Unisole F-200, 3.2 mm × 2.1 m). The analytical conditions were as follows: column oven temperature, 140°C; injector temperature, 210°C; detector temperature, 250°C. Flieg's scores, used to assess silage quality, were determined from LAC and VFA (Japanese Grassland Agriculture and Forage Seed Association, 2001).

2.1.2.4. Cultures and incubations

The TMR silages, ground into 0.5-mm powders, were used as substrates for *in vitro* cultures.

Two adult wethers (average initial body weight, 78.5 kg) fitted with rumen cannulae were used as donors of ruminal fluid. The wethers were fed basal diets of 50% reed canary grass (*Phalaris arundinacea* L.) hay and 50% commercial feed concentrate (Koushi-Ikusei-Special; Kitanihon-Kumiai-Feed, Miyagi, Japan) at maintenance energy

level (20 g DM/kg body weight daily) and had free access to clean drinking water. They were fed once daily at 09:00 h and were cared for according to the animal care and use guidelines of the Faculty of Agriculture, Yamagata University.

Rumen fluid was collected through the rumen cannulae 2 h after feeding and diverted to plastic bottles. These fluids were filtered through four layers of cheesecloth and then combined on an equal volume basis. The compound filtrate was mixed with CO_2-bubbled McDougal's artificial saliva (pH 6.8; McDougal, 1948) at a ratio of 1:4 (v/v). Then 50 ml diluted rumen fluid were transferred to 128-ml serum bottles containing 0.5 g ground TMR, and flushed with O_2-free CO_2. The tubes were capped with a butyl rubber stopper and sealed with an aluminum cap. Incubations were performed in triplicate at 39°C for 6 h in a water bath with a reciprocal shaker (100 strokes/min).

2.1.2.5. Analysis of fermentation productions

To terminate fermentation at the end of incubation, 25 μL of formaldehyde solution (35%) were injected into serum bottles, which were immediately sealed and cooled at room temperature. Gas samples were collected by air syringe from the serum bottles and injected into a gas chromatograph (GC323; GL Sciences, Tokyo, Japan) equipped with a thermal conductivity detector and stainless used steel column (WG-100 SUS 1.8 m ×6.35 mm OD), and the methane production in each serum bottle was measured. The analytical conditions were as follows: column oven temperature, 50°C; injector temperature, 50°C; detector temperature, 50°C.

2.1.2.6. In vitro DM digestibility, and methane and VFA production

Separate sub-samples of the supernatant were taken to determine the pH and VFA concentration. The bottles were rinsed with warm water to remove all solid residues, which were then oven-dried at 60°C and stored for further analyses. In total, 2 g of dried

residue were oven-dried at 135°C and stored to determine DM digestibility (Horii et al., 1971).

2.1.2.7. Statistical analyses

Analyses were performed using the general linear model procedure (SAS institute, Cary, NC, USA). Data on fermentative characteristics, *in vitro* DM digestibility, and ruminal methane and VFA production of TMR silages were subjected to a one-way ANOVA. Tukey's test was used to identify differences ($P < 0.05$) between means.

2.2.3. Results

2.2.3.1. Chemical composition of materials and silage

As shown in Table 2.2.1, the content of DM, CP, EE, NFC, ash, and NDF in molasses was 72.7, 4.3, 0.7, 83.6, 11.4, and 0%, respectively (Wang and Goetsch, 1998; National Agricultural Research Organization, 2001). The WCR had slightly less organic matter (OM) and CP than the yellow-ripe-stage WCR reported by Cai (2003). The content of CP, NFC, and NDF in the TC was 30.1, 15.8, and 37.7%, respectively.

There were no significant differences in chemical composition between the control and M-LAB silages, although the DM, CF, NFC, and NDF of the latter were 3.1% higher, 1.2% lower, 1.1% higher, and 1.8% lower, respectively (Table 2.2.3).

2.2.3.2. Fermentation quality

As indicated by the low pH values (4.03 and 3.99) and NH_3-N/TN content (2.83 and 2.97%), high LAC (3.42 and 3.06%), and high V-scores (99.8 and 99.8) for silages with and without M-LAB, the two TMR silages were well preserved (Table 2.2.4). Although the levels of moisture, pH, acetic acid, propionic acid, butyric acid, and NH_3-N/TN, and the V-scores, did not differ significantly, LAC did ($P < 0.05$; 3.06 and 3.42% for control

and M-LAB silages, respectively).

2.2.3.3. In vitro DM digestibility and production of methane and VFA

After 6 h of incubation of TMR silages, *in vitro* DM digestibility and methane production were 5.6% higher and 2.0% lower in M-LAB silage, respectively. There were no other significant differences among any characteristics, although acetic and butyric acid levels were higher and lower, respectively, in M-LAB silage.

2.2.4. Discussion

In the previous experiment (described above), silages with 30% TC had the highest LAC. Therefore, to further increase LAC, molasses was used in combination with TC. Previous reports suggested that molasses is a fermentable carbohydrate (Maiga and Schingoethe, 1997), and many researchers (McDonald and Purves, 1956; Archibald et al., 1960; Alli et al., 1984) have reported its successful use with grass silage. In addition, molasses is a food byproduct of sugar beet and sugarcane production. Although molasses with high soluble carbohydrates is used as a major energy source for meat or milk production (Preston, 1982; Yan et al., 1996; Wang and Goetsch, 1998; Fadel, 1999; Araba et al., 2002; Granzin and Dryden, 2005; Shellito et al., 2006; Sahoo and Walli, 2008), it has not been used as an additive to TMR silage with WCR.

Alli et al. (1984) assessed the effects of molasses on the fermentation of chopped whole-plant *Leucaena*. Silages were treated with molasses at a rate of either 2.25% or 4.5% fresh weight and 4.5% *Leucaena* at the time of ensiling, which led to increased rates of lactic acid production, lower pH, decreased DM loss, and reduced levels of volatile nitrogen, compared to *Leucaena* to which no molasses was added. In the present experiment, silages were treated with or without molasses at the rate of 4% fresh weight.

Although the addition of molasses did not significantly influence the chemical composition of the WCR TMR silage, it did increase DM and NFC by 3.06 and 3.56%, respectively. Similar to Alli et al. (1984), this was likely due to a high content of water-soluble carbohydrates that decreased DM loss and added NFC to the silage.

Adding molasses did not decrease pH, but did significantly increase LAC ($P < 0.05$), probably because LAB was also added to the silage, which may have led to the availability of more fermentable sugars for conversion to lactic acid, and also decreased pH, thereby inhibiting the activity of other bacteria (Cai, 2001; Cai et al., 2003). Also similar to Alli et al. (1984), adding molasses reduced the NH_3-N of silage by 4.9%, indicating reduced proteolysis, which could result from reduced activity of yeasts and moulds as well as reduced enzyme activity (Lanigan, 1961; Caipintero et al., 1969).

DM digestibility is higher in silage with LAB than without LAB because LAB reduces DM loss in silage fermentation (Cai, 2001; Cai et al., 2003). Furthermore, although there are some reports that adding molasses has no effect on DM digestibility in ruminants (Wang and Goetsch, 1998; Granzin and Dryden, 2005), many more studies (Hatch and Beeson, 1972; Chen et al., 1981; Wing et al., 1998; Fadel et al., 2000; Shellito et al., 2006; Sahoo and Walli, 2008) have reported that diets with molasses have higher ruminal DM digestibility. In the present experiment, there was a non-significant increasing trend in DM digestibility with M-LAB addition. Although not significant, methane production and the molar proportion of propionic acid decreased by 2.0% and increased by 1.4%, respectively, with M-LAB addition, which seems to be consistent with previous reports (Leng, 1970; Moss et al., 2000). Adding molasses, which has a high sugar content, may augment methane production in the rumen (Hindrichsen et al., 2005), explaining why, even though the two silages differed in LAC, there was no notable

difference in methane production. As dietary proportions of soluble carbohydrates such as molasses increase, the bacteria and protozoa populations shift in proportion to the VFA produced (Wing et al., 1998); for example, sucrose reduces acetic acid and increases butyric acid in rumen fluid (Waldo and Schultz, 1960), as does glucose and fructose but not xylose or arabinose (Sutton, 1968). Furthermore, reducing the proportion of propionate increases butyrate via a rapid increase in lactic acid production (Kellogg and Owen, 1969). Sucrose and molasses produce similar effects, and molasses may influence fermentation patterns via its sucrose content, because sucrose is the primary energy source in molasses (Kellogg and Owen, 1969). Wing et al. (1998) found that when 6% molasses (FM basis) was added to diets, ruminal acetic acid increased, while propionic acid and butyric acid changed only slightly. In the present experiment, adding 4% M-LAB (FM), increased and decreased acetic acid and butyric acid ($P < 0.05$), respectively, and slightly increased ($P = 0.203$) propionic acid. The A/P ratio tended to increase ($P = 0.07$) because of the high proportion of acetic acid.

2.2.5. Summary

The present experiment compared the effects of M-LAB on the quality of WCR TMR silages by measuring chemical composition and organic acids, and determining *in vitro* DM digestibility and the production of ruminal methane and VFA. TMR silages were prepared using WCR, feed concentrate, beet pulp, vitamin–mineral supplement, and TC, and the calculated content of CP and TDN were 16.4 and 75.1, respectively. Two experimental groups were prepared, namely, with or without M-LAB (4% M; 5 ppm *Chikuso-1*). After 60 days of fermentation, there were no notable differences in chemical composition between the silages, although the DM and NFC of M-LAB silages were

3.1% and 1.1% higher than those of control silages, respectively. Both silages had low pH and NH_3-N, high LAC and V-scores, and were of good quality. M-LAB silage had more lactic acid. After 6 h incubation of TMR silages, *in vitro* DM digestibility and methane production of M-LAB silages were 5.6% higher and 2.0% lower than silages without M-LAB, respectively. Total VFA and the molar concentration of propionic acid, isovaleric acid, and valeric acid did not differ between the two silages, while acetic acid and butyric acid were higher and lower, respectively, in M-LAB silage. Adding molasses increased the LAC of WCR TMR silage, but this did not significantly decrease ruminal methane production or increase propionic acid content.

Table 2.2.1. Chemical composition of WCR[1], concentrate, beet pulp and tofu cake used in TMR[2] silages

	WCR	Concentrate[3]	Beet pulp	DTC[4]	Molasses
DM[5] (%)	36.0	88.2	90.7	91.3	72.7*
CP[6] (% DM)	5.3	16.7	8.4	30.1	4.3*
EE[7] (% DM)	2.2	3.8	0.7	12.2	0.7*
NFC[8] (% DM)	32.1	60.1	33.7	15.8	83.6
Ash (% DM)	13.5	5.1	5.1	4.3	11.4*
ADF[9] (% DM)	30.2	8.7	25.6	22.2	-
NDF[10] (% DM)	48.0	14.4	52.1	37.7	0**

* National agricultural Research Organization (2001).

** Wang, Z.S. and Goetsch, A.L. (1998).

[1] Whole crop rice.

[2] Total mixed ration.

[3] Formula feed ("Koushi Ikusei Special Mash" made by Zenno with 120g/kg CP in fresh matter).

[4] Dry tofu cake.

[5] Dry matter.

[6] Crude protein.

[7] Ether extract.

[8] Nonfibrous carbohydrate(100 – CP – EE – NDF – ash).

[9] Acid detergent fiber.

[10] Neutral detergent fiber.

Table 2.2.2. Ingregient and nutrient composition of TMR[1] silage

	Treatment	
	Control	M-LAB
Ingredient		
Molasses (% FM[2])	—	4
Lactic acid bacteria (ppm FM)	—	5
WCR[3] (% DM[4])	30	30
Concentrate[5] (% DM)	25	25
Vitamin-mineral supplement[6] (% DM)	1.5	1.5
Beet pulp (% DM)	13.5	13.5
DTC[7] (% DM)	30	30
Nutrient composition		
CP[8] (% DM)	16.4	16.4
TDN[9] (% DM)	75.1	75.1

[1]Total mixed ration.

[2]Fresh matter.

[3]Whole crop rice.

[4]Dry matter.

[5]Formula feed ("Koushi Ikusei Special Mash" made by Zenno, TDN: 70.0%, CP:12.0% in fresh matter).

[6]Commercial vitamin-mineral supplement product(Snow brand seed, Iwate, Japan).

[7]Dry tofu cake.

[8]Crud protein.

[9]Total digestible nutrents.

Table 2.2.3. Chemical composition of TMR[1] silage

	Treatment		SEM[3]	P-value
	Control	M-LAB[2]		
DM[4]	35.9	37.0	1.85	0.6991
Organic matter (% DM)	92.6	92.3	0.19	0.3579
Crude protein (% DM)	15.3	15.1	0.26	0.7415
Ether extract (% DM)	5.1	5.6	0.19	0.1112
Nitrogen free extract (% DM)	57.1	57.5	0.56	0.6620
Crude fiber (% DM)	15.7	14.5	0.67	0.4160
NFC[5] (% DM)	30.9	32.0	0.85	0.4593
Crude ash (% DM)	7.4	7.7	0.19	0.3579
Acid detergent fiber (% DM)	19.0	20.2	0.70	0.4679
Neutral detergent fiber (% DM)	41.4	39.6	0.65	0.1389

[1]Total mixed ration.

[2]molasses-Lactic Acid Bacteria (Lactobacillus plantarum)

[3]Standard error of means.

[4]Dry matter.

[5]Non-fiberous carbohydrate (100 – CP – EE – NDF – CA).

Table 2.2.4. Fermentative characteristics of TMR[1] silages

	Treatment		SEM[3]	P value
	Control	M-LAB[2]		
Moisture (%)	64.1	63.0	0.950	0.6991
pH	3.99	4.03	0.030	0.4860
Lactic acid (% FM[4])	3.06[a]	3.42[b]	0.045	0.0083
Acetic acid (% FM)	0.09	0.09	0.003	0.7415
Propionic acid (% FM)	0.0	0.0	0.000	NS[6]
Butyric acid (% FM)	0.0	0.0	0.000	NS
NH_3-N/TN[5] (% FM)	2.97	2.83	0.143	0.5970
V-score	99.8	99.8	0.010	0.7229

[1]Total mixed ration.

[2]molasses-Lactic Acid Bacteria (Lactobacillus plantarum).

[3]Standard error of the mean.

[4]Fresh matter.

[5]Total nitrogen.

[6]Nosignificant.

[a,b]Means within rows with different letters differ ($P < 0.05$).

Table 2.2.5. Measurements of dry matter digestibility, methane production and VFA concentration *in vitro* after 6 hours incubation of TMR[1] silages

	Treatment		SEM[3]	P value
	Control	M-LAB[2]		
DM[4] digestibility (%)	42.15	44.50	0.97	0.1950
Methane production (L/kg DDM[5])	10.46	10.25	0.18	0.4757
Total VFA (mmol/dL)	5.29	5.70	0.24	0.3974
Acetic acid (A) (mol%)	36.99[a]	38.79[b]	0.35	0.0228
Propionic acid (P) (mol%)	39.95	40.50	0.24	0.2030
Butyric acid (mol%)	19.93[a]	17.80[b]	0.35	0.0189
Isovaleric acid (mol%)	0.36	0.32	0.05	0.6363
Valeric acid (mol%)	2.77	2.6	0.08	0.2081
A/P[6]	0.93	0.96	0.01	0.0784

[1]Total mixed ration.

[2]molasses-Lactic Acid Bacteria (Lactobacillus plantarum)

[3]Standard error of the mean.

[4]Dry matter.

[5]Digestible dry matter.

[6]Acetic acid/Propionic acid.

[a,b]Means within rows with different letters differ ($P < 0.05$)

Chapter 3

Effect of adding food by-products on the *in situ* and *in vivo* feed characteristics of TMR with WCR

3.1. Introduction

Along with a decrease in rice consumption among the Japanese people, the importance of rice production for feed has increased in terms of paddy field conservation and levels of feed self-sufficiency. Whole crop rice (WCR) is a suitable feed crop because it can withstand the heat and rain of Japanese summers, and harvesting machines as well as species adaptable to local cultivation and climate conditions have been developed. However, WCR silage used as feed is of poor quality due to its low lactic acid content resulting from a low level of soluble carbohydrates (Cai et al., 2003).

Many food and beverage by-products result from processing and manufacturing, but most is burned, dumped into landfills or used as compost, which leads to wasted resources, and possible environmental problems due to unsuitable disposal. Demand is increasing for efficient use of food by-products due to economic and environmental concerns. Food by-products such as tofu cake (TC), rice bran (RB) and wet green tea waste (WGTW) are high in crude protein (McPherson et al.), fatty acids, tannins and vitamins (Xu et al., 2001; Amissah et al., 2003; Kondo et al., 2004b; De Campos et al., 2007; Xu et al., 2007). Not only could these by-products be utilized as a source of nutrients for ruminants, but using them to replace imported commercial feedstuffs could save energy in transportation, and possibly reduce the environmental impact of burning them as waste or burying them landfills.

If WCR were ensiled with these food by-products as a TMR, this silage could have a high lactic acid content and be of good quality, and unpalatable by-products could be incorporated into the TMR if their odors and flavors were altered by fermentation. Research has been limited on effects of added food by-products in WCR TMR silage on silage fermentation and the feeding value of the silage for ruminants. If added food by-products result in more heterolactic silage fermentation, production of short-chain antifungal volatile fatty acids (VFA), along with lactic acid, would benefit silage fermentation quality and, possibly, animal intake. Moreover, using these food by-products and WCR as feed would be more efficient.

The objective of this study was to determine the fermentation characteristics of a TMR silage prepared from WCR and various food by-products (i.c., DTC, RB, WGTW) and the nutritive value of the silage for sheep as assessed by intake, *in situ* degradation and *in vivo* digestibility, preference and rumen fermentation.

3.2. Materials and Methods

This animal experiment was conducted with permission from the Committee of Animal Experimentation under the institutional guidelines for animal experiments at the Faculty of Agriculture, Yamagata University, and according to the animal care and use guidelines of the National Institute of Livestock and Grassland Science of Japan.

3.2.1. Silage preparation

The WCR (Haenuki) was cultivated using conventional methods in the paddy field of the Yamagata University Farm, harvested at the full-ripe stage and cut to a length of 2 cm. As shown in Table 3.1, TMR was prepared using a compound feed (Kitanihon-Kumiai Feed, Yamagata, Japan); WCR; dried beet pulp; a vitamin–mineral

supplement (Snow Brand Seed, Iwate, Japan; vitamin A, 5,000,000 IU/kg; vitamin D3, 1,000,000 IU/kg; vitamin E, 2 g/kg; vitamin K3, 0.2 g/kg; vitamin B1, 0.5 g/kg; vitamin B2, 1 g/kg; vitamin B6, 0.1 g/kg; vitamin B12, 0.001 g/kg; nicotinic acid, 6 g/kg; choline chloride, 2 g/kg; calcium pantothenate-D, 10 g/kg; Mn, 0.16 g/kg; Zn, 0.7 g/kg; Fe, 0.55 g/kg; Cu, 0.14 g/kg; I, 0.33 g/kg; Co, 0.04 g/kg; methionine, 1 g/kg; lidocaine hydrochloride, 0.5 g/kg); and a food by-product, either dry tofu cake (DTC), RB or WGTW. The DTC (40 kg) was obtained from Zenno, Tsuruoka, Japan; RB (40 kg) was obtained from Yamagata University Farm, Yamagata, Japan; and WGTW (160 kg) was obtained from a commercial beverage factory (Marubishi Food, Yamagata, Japan) and transported to the laboratory. The DTC and RB were stored at room temperature for 2 days, while WGTW was stored (4°C) for 2 days before being used. The TMR ingredient proportions are in Table 3.2. Treatments included a control silage (i.e., no food by-product added) and addition of DTC, RB or WGTW at 30 % of TMR DM each. Moisture was adjusted with water to 55 %. The ensiled amounts of TMR were 100 kg each in drum can silos of 200 L in volume (Minikon-silo; KD-Service, Tokyo, Japan), and the same TMR was ensiled in two drum can silos, which were stored outdoors (9°C-32°C) for 60 days of fermentation.

3.2.2. In situ incubation study

3.2.2.1. Animals and feeding

The *in situ* degradabilities of feed were measured in two Japanese black steer (body weight, 418 ± 2.8 kg) fitted with rumen cannulae. The animals were fed 25% DM commercial feed concentrate (Koushiikusei-Special; Kitanihon-Kumiai-Feed, Miyagi, Japan), 25% DM RB (Yamagata University Farm, Yamagata, Japan), and 50% DM reed canary grass (*Phalaris arundinacea L.*) hay at 1.4% body weight. The animals were fed

twice daily, at 09:00 and 16:00 h, in equal amounts. Water and mineral blocks (Koen-S, Nippon Aenyaku Kogyo Co. Ltd., Fukushima, Japan) were made available at all times.

3.2.2.2. Nylon bag incubation

The *in situ* degradation of DM, CP, and NDF in WCR TMR silages with and without food by-products was determined. The samples were dried in a forced draft oven at 60°C for 48 h, ground into a 2-mm powder, and mixed in two drum can silos for each treatment. The surface area and pore size of the nylon bags were 100 mm × 200 mm and 50 μm, respectively, and each bag contained 5 g of composite sample. Fourteen bags of each treatment per animal were prepared. Bags were incubated in each animal (in reverse order between the animals) for 3, 6, 12, 24, 48, and 72 h, and two bags of each treatment were removed from each animal at a common time. After removal, all nylon bags were immersed in ice water for 10 min to stop microbial activity. 0-h bags were washed at 39°C for 30 min in a water bath with a reciprocal shaker (100 strokes/min). All bags were then washed in a washing machine for 10 min and agitated for 3 min prior to draining. The bags were washed again by hand with cold tap water and then dried at 60°C for 48 h. Dried samples were used to analyze DM, CP, and NDF content. The mean of the measurements from two bags from each steer was used to calculate values for ruminal disappearance.

3.2.3. Digestion study

Four Suffolk sheep (26.9 ± 1.4 kg) were used in a 4 × 4 Latin square design experiment. The sheep were individually housed in metabolic cages and fed the four silages at 2.5 % of their body weight (BW) on a DM basis once daily at 09:00 h. The metabolizable energy maintenance level was set according to *Nutrient Requirements of Sheep* (NRC, 1985). Water was accessible at all times. A 7 day preliminary adjustment

period was followed by a 5 day period during which all feces and urine were collected. The digestibility and nutrient content of each food by-product were calculated by the methods of National Agricultural Research Organization (2001).

Blood samples were also collected by venipuncture of jugular vein 0, 2 and 4 h after the morning feeding on day 12 of each period and received into plain vacutainer tubes (VENOject II; TERUMO, Tokyo, Japan). Hematocrit values were determined in duplicate by using micro-hematocrit tubes and centrifuging in a 12000 rpm Centrifuge (HEMATO CRIT; KOKUSAN, Tokyo, Japan) for 5 min (Dacie and Lewis, 1975) and plasma was prepared by centrifugation (12000 rpm, 20 min, 4 °C) and stored at -20°C until glucose and urea-N were determined.

Ruminal fluid was sampled immediately before the morning feeding and at 2 and 4 h after feeding on Day 13 of each period. Ruminal fluid pH was measured immediately, and samples were separated from feed articles through two layers of gauze. Samples were stored frozen (–20°C) for later analysis of VFA and NH_3-N.

3.2.4. Preference study

Four Suffolk sheep (27.4 ± 2.0 kg) were used to measure silage preference. For free choice, two kinds of TMR were put in separate containers and simultaneously offered for 15 min twice daily at 09:00 and 15:00 h. The quantities were adequate so that a surplus remained after 15 min. Refusals were weighed after 15 min, and intake quantities were calculated. This procedure was repeated for every combination of two silages.

3.2.5. Chemical and microbial analysis

The WCR, WGTW and TMR silages and feces were dried in a forced draught oven at 60°C for 48 h and ground to pass a 2 mm screen with a sample mill (Foss Tecator; Akutalstuku, Tokyo, Japan). The DM, CP, ether extract (EE), and ash were analyzed

according to methods 934.01, 976.05, 920.39, and 942.05, respectively, of the Association of Official Analytical Chemists (AOAC, 1990). The organic matter (OM) was calculated as weight loss upon ashing. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to Van Soest et al. (1991). Heat-stable amylase and sodium sulphite were used in the NDF procedure, and results are expressed without residual ash. Urinary N was determined using the Kjeldahl procedure previously described by the AOAC (1990). The gross energy (GE) was determined by using an automatic bomb calorimeter (CA-4PJ, Shimadzu, Kyoto, Japan).

Fermentation products of the silages were determined from cold water extracts. Wet silage (50 g) was homogenized with 200 ml sterilized distilled water and stored at 4°C overnight (Cai et al., 1999). The pH was measured using a glass electrode pH meter (Horiba D-21; Horiba, Kyoto, Japan). Lactic acid and NH_3-N were analyzed according to Takahashi et al. (2005). The VFA was steam-distilled and measured qualitatively and quantitatively using gas chromatography (G5000-A; Hitachi, Tokyo, Japan) equipped with a thermal conductivity detector and a glass column (Unisole F-200, 3.2 mm × 2.1 m). The analytical conditions were as follows: column oven temperature, 140°C; injector temperature, 210°C; detector temperature, 250°C. To assess the quality of the silage, we calculated the Frieg's mark from the lactic acid and the VFA concentrations and V-score from the NH_3-N/total N and VFA concentrations (Takahashi et al., 2005). The pH, VFA, and NH_3-N concentrations in ruminal fluid samples were measured using the same methods as for TMR silage filtrates.

Glucose and urea-N in serum were determined by using Glucose C II-testwoko (Wako Pure Chemical Industries, Osaka, Japan) and Urea-N B-testwoko (Wako Pure Chemical Industries, Osaka, Japan).

3.2.6. Calculations and statistical analysis

Values of ruminal disappearance of DM, CP, and NDF versus time were fitted to the exponential equation of Orskov and McDonald (1979): $P = a + b\,(1 - e^{-ct})$, where P is the disappearance rate at time t, a is the rapidly degradable DM, CP, or NDF fraction in the rumen, c is the rate constant of degradation of b, and t is the time of incubation. Curves were fitted with the NLIN procedure of SAS (1988) to estimate the rapidly (a) and slowly degradable (b) fractions, and rate of degradation (c). Effective degradability (ED) was calculated as a function of the fractional rate of degradation and the fractional rate of passage (k_p), assuming a constant value of 0.05 h^{-1}: $ED = a + b\,[(b \times c)/(c + k_p)]$.

The chemical composition and fermentative characteristics of TMR silages, and the estimated constant values of the exponential equation and ED from the *in situ* incubation experiment, were analyzed using a one-way ANOVA. Digestion data were analyzed as a 4 × 4 Latin square using the General Linear Model procedure of SAS (1995), with diet, period, and animal included in the model. Tukey's test (SAS, 1995) was used to identify differences ($P < 0.05$) between means.

3.3. Results

3.3.1. Chemical composition of materials and silage

As shown in Table 3.1, the organic matter (OM) and CP of the WCR were all slightly less than those in the WCR (Hamasari and Kusahonami) of the yellow-ripe stage as reported by Cai (2003). Among the three food by-products, DM and nonfibrous carbohydrate (NFC) were lowest in WGTW. Levels of CP, ADFom, and aNDFom in DTC and WGTW were higher than in RB, whereas levels of EE, NFC, and crude ash were lower.

No notable differences occurred in DM or NDF, but CP, EE and gross energy increased ($P < 0.05$) with addition of food by-products (Table 3.3), whereas NFC decreased ($P < 0.05$). The ADFom for WGTW silage was higher ($P < 0.05$) than for the control. In addition, OM and CP for both DTC and WGTW silage were higher ($P < 0.05$) than for RB silage, while EE for RB silage was higher ($P < 0.05$) than for either DTC or WGTW silage.

3.3.2. Fermentation quality

The silages were well preserved, as indicated by low pH values and NH_3–N/TN contents, high lactic acid contents, as well as high Frieg's marks and V-scores (Table 3.4). Although levels of moisture and acetic acid, Frieg's marks, and V-scores did not differ, pH values, lactic acid concentrations, and NH_3–N/TN contents did ($P < 0.05$), with pH values ranging from 3.93 to 4.04. The lactic acid contents in the control, DTC, RB and WGTW silage were 2.44, 3.27, 3.08 and 2.49 %, respectively. The lactic acid content for the DTC silage in particular was higher ($P < 0.05$) than for the WGTW silage and control. The NH_3–N content was lowest in the DTC and WGTW silages. Propionic and butyric acids were found in trace quantities in each of the four silages.

3.3.3. In situ degradability

The *a*, *b*, and *c* fractions and ED data are presented in Table 3.5. TC-treated silage had a lower *a* and *b* of DM than controls, RB, and WGTW ($P < 0.05$); the food-byproduct treatment had no effect on *c* or $a + b$ of CP; and RB-treated silage had a higher ED of DM than the TC and WGTW ($P < 0.05$). The *a* of CP was highest in RB, followed by controls, TC, and WGTW, and there was a significant difference among the four silages ($P < 0.05$). The *b* of CP for both RB and control silages were lower than for TC or WGTW silage ($P < 0.05$), and the WGTW silage was the highest among the four silages ($P < 0.05$). The RB

and WGTW silages had a lower *c* of CP than controls ($P < 0.05$), and TC silage had a higher $a + b$ of CP than controls but a lower ED of CP than controls and RB ($P < 0.05$). TC-treated silages had a lower *a* but higher *b* of NDF than controls, RB, and WGTW ($P < 0.05$), and RB and WGTW silage also had a higher *b* of NDF than controls ($P < 0.05$). The food-byproduct treatments had no effect on *c* or $a + b$, although TC and WGTW silage had a lower ED of NDF than controls ($P < 0.05$).

The changes in DM, CP, and NDF degradability over incubation time are given in Figure 3.1. All components in all silages rapidly degraded in the rumen for the first 24 h, after which they tended to degrade more slowly until 72 h. The degradability of all components of TC silage was lower than controls and RB silages at 21 h but thereafter was higher than RB and WGTW silage. The CP degradability of WGTW silage was always the lowest among the four silages.

3.3.4. In vivo nutrient digestibility and nitrogen retention

Apparent digestibility of the DTC silage was higher than both the RB and WGTW silages (Table 3.6). Remarkable differences were observed in DM, OM, CP, EE, CF and NDF between the DTC and RB silages, and in the digestibility of all nutrients except for CP between the DTC and WGTW silages. Digestible CP was higher ($P < 0.05$) in both the DTC and WGTW silages than in the RB silage, while digestible energy was higher ($P < 0.05$) in both the DTC and RB silages than in the WGTW silage. Total digestible nutrients (except for digestible CP) and digestible energy were higher ($P < 0.05$) in both the DTC and RB silages than in the WGTW silage.

To determine the digestibility of DTC, RB, and WGTW, we prepared the control treatment as a basal diet. The ratio of concentrate to forage was less than 60 % (DM), but CP content (11.5 % DM) in control was slightly lower compared with the requirements

for sheep (National Research Council, 1985). In the present study, not only was the digestibility of DTC higher than that of RB and WGTW (Table 3.7), but the digestibility of the DTC silage was higher than that of the RB and WGTW silages.

3.3.5. Nitrogen retention

Nitrogen intake of the DTC silage was highest among the three food by-product treatments (Table 3.8), and together with the WGTW silage was higher ($P < 0.05$) than in the RB silage. Fecal excretion of nitrogen was similar among the DTC, RB, and WGTW silages, but a significant difference was detected between the WGTW silage and the control. Urinary excretion of nitrogen and nitrogen retention in the DTC (7.44 and 6.19 g/day, respectively) and WGTW (5.78 and 6.45 g/day, respectively) silages were higher than in the RB silage (4.47 and 4.64 g/day, respectively), but urinary excretion of nitrogen, nitrogen retention, and allantoin were not significantly different among the treatments with added food by-products.

3.3.6. Ruminal fermentation

Ruminal pH and VFA concentrations were not affected by dietary silage (Table 3.9). Molar proportions of acetic acid in the DTC and RB silages were lower ($P < 0.05$) than those in the WGTW silage or in control before feeding. Two hours after feeding, the DTC silage had lower ($P < 0.05$) acetic acid concentrations than the WGTW silage and, 4 h after feeding, the DTC, RB and control silages had lower ($P < 0.05$) acetic acid concentrations than the WGTW silage. Molar proportions of propionic acid in the RB silage were higher ($P < 0.05$) than in the WGTW silage before feeding. Molar proportions of isobutyric acid, isovaleric acid, and valeric acid were differed before feeding among the four treatments. The ratio of acetic acid to propionic acid (A:P) of the RB silage was lower ($P < 0.05$) than that of the WGTW silage before feeding, but there was no

difference among the four treatments after feeding. Before feeding, the DTC silage had higher ($P < 0.05$) NH_3–N concentrations than the RB silage or control, and the WGTW silage had higher ($P < 0.05$) NH_3–N concentrations than the RB silage. Although 2 h after feeding, NH_3–N concentrations in the rumen fluid of sheep fed DTC silage were highest ($P < 0.05$), the DTC silage still had higher ($P < 0.05$) NH_3–N concentrations than the RB silage and control 4 h after feeding and the WGTW silage had higher ($P < 0.05$) NH_3–N concentrations than the control.

3.3.7. Blood

The hematocrit, glucose, and urea-N levels in the blood plasma of steer fed TMR silages are shown in Table 3.10. Neither hematocrit nor glucose was notably different among the four silages. Urea-N for TC-treated silage was higher ($P < 0.05$) than for controls, and 2 h after feeding was the highest ($P < 0.05$) among the four silages. Urea-N for RB silage 2 h after feeding was also higher than for controls, whereas that for WGTW silage was higher than controls before feeding and 2 h after feeding. Hematocrit, glucose, and urea-N were always within normal values for steer.

3.3.8. Preference

When the DTC silage was fed to sheep, together with RB, WGTW or control silage, the relative intake ratios were 0.683, 0.613 and 0.718 (DM), respectively (Table 3.11). The mean relative intake ratios for control, DTC, RB and WGTW silage were 0.485, 0.671, 0.397 and 0.447, respectively, and the rank order of preference was DTC, control, WGTW and RB silage. Preference was highest for DTC silage and lowest for RB silage.

3.4. Discussion

Cai et al. (2003) and Ennahar et al. (2003) reported that the low content of soluble

carbohydrate in WCR resulted in poor fermentation quality of WCR silage because there is not enough soluble carbohydrate available for complete consumption of sugar by action of lactic acid bacteria, and yeasts are inhibited. Therefore, a need exists to improve the soluble carbohydrate content of silage materials with addition of food by-products and other materials, and to better understand the ensiling characteristics of WCR TMR silage with added food by-products. In this way, current technologies can be used to better prepare silage and more efficiently use food by-products. Having studied the feeding techniques of TC for TMR silage of dairy cows, Ide (2002) reported that the mixing ratio of DTC in TMR is suitable at 11 %. Yokota and Ohshima (1997) reported that Napier grass ensiled with 15 % RB is a good quality silage. Xu et al. (2007) reported that TMR silage with 15 % (DM) WGTW has a higher lactic acid content than that with 10 % (DM) added WGTW. In the present study, food by-products were added at 30 % (DM), and lactic acid content and CP ranged from 2.44 % (FM) to 3.27 % (FM) and 11.5 % (DM) to 15.3 % (DM), respectively.

Cai et al. (2001) and Xu et al. (2003) reported that during commercial tea production, tea leaves are soaked in 90°C water resulting in very low lactic acid bacteria (LAB) and water-soluble carbohydrate contents of the WGTW. This might be one reason that lactic acid content in the WGTW silage was lower than in the DTC or RB silages. Takahashi et al. (2005) reported that good-quality silage is produced from materials with high concentrations of water-soluble carbohydrates, and Yang (2004) reported that the addition of NFC to Napier grass at ensiling is beneficial to silage fermentation quality. In the present study, NFC content in the control was higher, but lactic acid concentration lower, than in the other three treatments, suggesting that food by-products might have some chemical or biological effect on bacterial flora during ensiling (Kondo et al., 2004).

However, the mechanism underlying this phenomenon is not clear. Davies et al. (1998) reported that herbages with low concentrations of water-soluble carbohydrates produce inferior-quality silage having a high NH_3–N content. In the present study, although the control contained the highest NFC and lowest CP values, the NH_3–N ratio in the control was higher than in the DTC and WGTW silages. Xu et al. (2007) reported that addition of WGTW to TMR silage decreased the NH_3–N. However, the reason why the NH_3–N content in the DTC silage was lower than in the control is not clear.

RB degraded quickly in the rumen because it is a high-energy feed with high CP and EE content (Enishi and Kawashima, 2003). Therefore, RB-treated silage had the highest degradability for the first 24 h. Thereafter, however, TC silage had the highest degradability, which is consistent with the *in vivo* experiment. In addition, the degradability of WGTW silage was lower than the other three silages, due to its high NDF content (Xu et al., 2004a, 2007).

TC-treated silage increased the urea-N in the blood of animals due to its high CP content. However, urea-N levels remained within the normal range for steer (Kaneko, 1989).

Xu et al. (2001) reported that ingested TC will not alter ruminal fermentation. Forster et al. (1993) reported that factors that might contribute to the difference in OM digestibility between ground corn and RB include dietary levels of fat and starch and low digestibility of aNDFom in RB. Belyea et al. (1989) observed that only 33.0 % (DM) of aNDFom in RB was potentially digested *in vitro*, which could have accounted for most of the difference in the digestibility of DM or OM between DTC and RB. Furthermore, decreases in digestibility have been recorded after addition of sunflower oil to the diet of growing beef cattle (McGinn et al., 2004), and Jordan et al. (2006) observed a decrease in

digestibility of DM, OM, CP and gross energy in a diet with copra meal treatment. In our study, the digestibility of RB and RB silage was lower than that of DTC and DTC silage, respectively. These decreases occurred because of the higher EE content in RB (McGinn et al., 2004; Jordan et al., 2006) than in DTC. In addition, the high content of fatty acids in RB (Warren and Farrell, 1990), and the greater abundance of fatty acid adsorption sites to feed particles with diets that limit adherence to microbial cells, might also be reasons for the adverse effects of fat on digestibility of RB and RB silage (Ohajuruka et al., 1991). Although the reason for the lower digestibility of WGTW and WGTW silage compared to DTC or DTC silage is unclear, it may be the higher aNDFom content in WGTW than in DTC (Xu et al., 2004a). Furthermore, Xu et al. (2007) reported decreases in CP digestibility due to addition of WGTW. However, in our study, the digestibility of CP in the WGTW silage, together with DTC or DTC silage, was higher than for RB or RB silage.

In our study, ruminal pH was similar in sheep fed the four silages, although both DTC and WGTW had higher contents of CP than RB and the control. This is consistent with results by Ipharraguerre et al. (2005), who reported that varying the CP content of the diet of dairy cows does not affect ruminal pH, but increases in CP in the diet cause increases in ruminal NH_3–N concentrations, which was also observed in the sheep fed the DTC silage in the present study. Although CP content in the WGTW silage was similar to that in the DTC silage, NH_3-N concentrations in the ruminal fluid of sheep fed the WGTW silage were different from those in the sheep fed the DTC silage due to decreases in CP digestibility attributable to tannins in the WGTW feed (McSweeney et al., 2001). No differences were observed in concentrations of total VFA in the ruminal fluid among the four silages, which is not consistent with results of Ipharraguerre et al. (2005), who

reported that the concentration of VFA in the ruminal fluid increases with a high CP content in the diet. Given this stable ruminal pH, and VFA concentrations, one may think that ruminal conditions were not affected by the lactic acid concentrations in our four silages. Ipharraguerre et al. (2005) reported that molar proportions of acetate and propionate are not affected by the concentration of dietary CP. In other experiments, increasing the CP content in the diet consistently enhanced the concentration of NH_3–N in the rumen, but not the molar proportions of branched-chain VFA (Cunningham et al., 1996). In our study, 4 h after feeding, the acetic acid concentration for WGTW was highest, which might have been attributable to the high fiber content of WGTW. This is consistent with Melaku (2004), who reported that the high fiber content of *Lablab purpureus*, and graded levels of *Leucaena pallida* 4203, contribute to the higher molar proportion of acetate compared to graded levels of *Sesbania sesban* 1198. Chan et al. (1997) reported that ruminal pH is unaffected by fat in diets, but that concentrations of NH_3–N and butyrate in ruminal fluid are increased by supplemental fat. In our study, the concentration of isobutyric acid in the ruminal fluid for RB silage was higher than for the other silages before feeding, and the difference between the RB and the WGTW silages and concentrations of NH_3–N in the ruminal fluid of sheep fed the RB silage was higher than for the control. This may have occurred because of the higher EE content in the RB silage. Ajisaka et al. (2002) reported that molar proportions of acetic acid remain unchanged with addition of medium-chain fatty acid-cyclodextrin, whereas those of propionic acid increase. In the present study, high molar proportions of propionic acid occurred in the ruminal fluid of sheep fed the RB silage before and after feeding, and there was a difference between the RB and WGTW silages before feeding. Moreover, high molar proportions of isovaleric acid and valeric acid occurred in the ruminal fluid of

sheep fed the RB silage. Busquet et al. (2005) reported that garlic oil decreases acetate acid and increases propionic acid, causing a decrease in the A:P ratio. In the present study, low A:P ratios of ruminal fluid in sheep fed the RB silage occurred before feeding, but only between the RB and WGTW silages.

Because DTC had a high CP content, it was considered that the high CP content in the DTC treated silage increased the urea-N in blood of animal, but it was within normal range (Kaneko, 1989).

Food selection by ruminants may involve interactions among the senses of smell and taste as well as satiety and malaise (Provenza, 1995). Differences in animal age also influence preference (Nombekela et al., 1994). As our study used animals of the same age and species, differences arising from age and species should have been avoided, since Baumont et al. (2000) reported that the relationship between the nutritive value of forages and voluntary intake is well established. Hadjigeorgiou et al. (2003) found that goats preferred grasses with higher digestibility, but lower aNDFom, ADFom and lignin. Alonso-Díaz et al. (2008) reported that goats appeared able to discriminate among feeds to select those with higher digestibility, and that goats might have evolved the ability to detect and select more digestible feeds to optimize their nutrient intake rate and, consequently, reduce predation exposure. In particular, differences in chemical and physical composition may affect the taste and texture of the food, and thus preference (Nombekela et al., 1994). Concentrations of NH_3–N and butyric acid in silage negatively impact the voluntary intake of grass silage fed to growing cattle (Krizsan and Randby, 2007). In our study, DTC silage was the most preferred because it was the most digestible of the four silages. The lower digestibility of DM, OM and aNDFom, and higher concentration of NH_3–N in the RB silage, might have contributed to the low preference

for this silage. The higher ADFom, lower digestibility of ADFom and aNDFom, and lower DE in the WGTW silage might explain why the preference for WGTW silage was lower than that for DTC or control silage.

3.5. Summary

Four sheep were used in a 4×4 Latin square design experiment to study fermentation quality, digestibility, and preference of a whole crop rice (WCR) total mixed ration (TMR) silage with food by-products. Experimental treatments included control silage (i.e., no food by-product) and 30 % each of TMR (DM) as dry tofu cake (DTC), rice bran (RB) or wet green tea waste (WGTW). Silages ensiled for 60 days were well preserved with low pH (<4.06) and NH_3–N contents, and high lactic acid content. A higher ($P <$ 0.05) content of lactic acid was observed in the DTC silage. In addition, compared to the control, the DTC and RB treatments increased the CP, EE and GE contents of the silages, and WGTW treatments increased the CP and ADFom contents of the silages. Preference was affected by inclusion of food by-products. The mean relative intake of TMR for the control, DTC, RB and WGTW silages were 0.485, 0.671, 0.397 and 0.447, respectively. Compared to the control, the DTC treatment increased silage preference, while RB and WGTW treatments decreased silage preference. No differences were observed among treatments in ruminal pH or total volatile fatty acid concentrations, but ruminal NH_3–N content was highest in the DTC silage (P = 0.0191) 2 h after feeding. The molar proportion of acetic acid in ruminal fluid was highest for the WGTW silage (P = 0.0004) 4 h after feeding. Molar proportions of propionic acid and isobutyric acid in ruminal fluid were higher for the RB silage (P = 0.0277 and P = 0.0368, respectively) than for the WGTW silage before feeding, and the molar proportions of isovaleric acid and valeric

acid in ruminal fluid were higher for the RB silage (P = 0.0183 and P = 0.0113, respectively) than for either the WGTW or control silage before feeding. Among the three food by-products, the digestibility of DTC was highest. Findings suggest that food by-products can be used in WCR TMR silage, and that the silages can be of good quality, and that silage with added DTC has high digestibility and good preference.

Table 3.1. Chemical composition of WCR[1], concentrate, beet pulp, tofu cake, rice bran, and green tea grounds used in TMR[2] silages

	WCR	Concentrate[3]	Beet pulp	DTC[4]	RB[5]	WGTW[6]
DM[7] (%)	36.0	88.2	90.3	91.3	89.4	17.2
CP[8] (% DM)	5.3	16.7	8.4	30.1	16.9	32.0
EE[9] (% DM)	2.2	3.8	0.7	12.2	24.5	5.4
NFE[10] (% DM)	52.8	69.8	68.5	40.2	37.5	40.5
CF[11] (% DM)	27.2	4.7	17.3	13.2	8.7	18.8
NFC[12] (% DM)	32.1	60.0	33.7	15.8	16.8	9.5
CA[13] (% DM)	13.5	5.1	5.1	4.3	12.4	3.3
ADF[14] (% DM)	30.2	8.7	25.6	22.2	12.8	32.1
NDF[15] (% DM)	48.0	14.4	52.1	37.7	29.5	49.8

[1]Whole crop rice.

[2]Total mixed ration.

[3]Formula feed ("Koushi Ikusei Special Mash" made by Zenno, TDN: 70.0%, CP:12.0% in fresh matter).

[4]Dry tofu cake.

[5]Rice bran.

[6]Wet green tea waste.

[7]Dry matter.

[8]Crude protein.

[9]Ether extract.

[10]Nitrogen-free extract.

[11]Crude fiber.

[12]Nonfibrous carbohydrate(100 – CP – EE – NDF – CA).

[13]Crude ash.

[14]Acid detergent fiber.

[15]Neutral detergent fiber.

Table 3.2. Ingredient proportions and nutrient composition of TMR[1] silage

	Treatment			
	Control	DTC	RB	WGTW
Ingredient				
WCR[2](% DM[3])	43	30	30	30
Concentrate[4](% DM)	36	25	25	25
Vitamin supplement[5](% DM)	2.0	1.5	1.5	1.5
Beet pulp(% DM)	19.0	13.5	13.5	13.5
DTC[6](% DM)	0	30	0	0
RB[7](% DM)	0	0	30	0
WGTW[8](% DM)	0	0	0	30
Nutrient composition				
CP[9](% DM)	11.2	16.4	12.8	17.0
TDN[10](% DM)	67.0	75.1	74.3	68.2

[1]Total mixed ration.

[2]Whole crop rice.

[3]Dry matter.

[4]Formula feed ("Koushi Ikusei Special Mash" made by Zenno, TDN: 70.0%, CP:12.0% in fresh matter).

[5]Commercial vitamin-mineral supplement product (Snow bran seed, Iwate, Japan).

[6]Dry tofu cake.

[7]Rice bran.

[8]Wet green tea waste.

[9]Crud protein.

[10]Total digestible nutrents.

Table 3.3. Chemical composition of TMR[1] silage

	Treatment				SEM[5]	P value
	Control	DTC[2]	RB[3]	WGTW[4]		
DM[6] (%)	41.5	42.6	43.0	43.1	0.26	0.0604
Organic matter (% DM)	91.9^{ab}	92.8^{a}	90.7^{b}	92.2^{a}	0.17	0.0169
Crude protein (% DM)	11.5^{c}	15.2^{a}	11.9^{b}	15.3^{a}	0.31	0.0008
Ether extract (% DM)	2.6^{c}	5.0^{b}	8.3^{a}	2.7^{c}	0.30	0.0009
Nitrogen free extract (% DM)	65.7^{a}	57.8^{b}	56.9^{b}	57.3^{b}	0.18	<0.0001
Crude fiber (% DM)	14.1	14.8	13.6	16.9	0.58	0.0586
NFC[7] (% DM)	35.1^{a}	29.6^{b}	28.5^{b}	29.1^{b}	0.94	0.0314
Crude ash (% DM)	8.1^{ab}	7.2^{b}	9.3^{a}	7.8^{b}	0.17	0.0169
Acid detergent fiber (% DM)	18.8^{b}	20.3^{ab}	18.7^{b}	23.5^{a}	0.54	0.0195
Neutral detergent fiber (% DM)	44.7	43.0	42.0	45.2	1.21	0.3900
Gross energy (kcal/g DM)	4.4^{b}	4.6^{a}	4.6^{a}	4.5^{ab}	0.02	0.0075

[1]Total mixed ration.

[2]Dry tofu cake.

[3]Rice bran.

[4]Wet green tea waste.

[5]Standard error of means.

[6]Dry matter.

[7]Nonfiberous carbohydrate (100 – CP – EE – NDF – CA).

[a,b,c]Means within rows with different letters differ ($P < 0.05$).

Table 3.4. Fermentative characteristics of TMR[1] silages

	Treatment				SEM[5]	P value
	Control	DTC[2]	RB[3]	WGTW[4]		
Moisture (%)	58.47	57.39	56.98	56.94	0.26	0.0604
pH	3.93[c]	4.04[ab]	4.06[a]	3.95[bc]	0.01	0.0121
Lactic acid (% FM[6])	2.44[b]	3.27[a]	3.08[ab]	2.49[b]	0.10	0.0154
Acetic acid (% FM)	0.19	0.18	0.20	0.17	0.01	0.6538
Propionic acid (% FM)	0.0	0.0	0.0	0.0	0.00	NS[8]
Butyric acid (% FM)	0.0	0.0	0.0	0.0	0.00	NS
NH_3-N/TN[7] (%)	4.20[a]	3.00[b]	4.51[a]	2.66[b]	0.12	0.0022
Frieg's mark	100	100	100	100	0.00	NS
V-score	100	100	100	100	0.00	NS

[1]Total mixed ration.

[2]Dry tofu cake.

[3]Rice bran.

[4]Wet green tea waste.

[5]Standard error of the mean.

[6]Fresh matter.

[7]Total nitrogen.

[8]Nonsignificant.

[a,b,c]Means within rows with different letters differ ($P < 0.05$).

Table 3.5. Constant values of the exponential regression equation for prediction the DM[1], CP[2] and NDF[3] degradability of TMR[4] silages.

	Treatment				SEM[8]	P value
	Control	DTC[5]	RB[6]	WGTW[7]		
DM						
a (%)	38.9^{f}	31.2^{e}	44.2^{f}	42.8^{f}	1.062	0.0003
b (%)	44.3^{f}	60.4^{e}	42.7^{f}	43.8^{f}	2.053	0.0008
c (h^{-1})	0.0469	0.0387	0.0360	0.0273	0.005	0.1683
a+b (%)	83.1	91.6	86.8	86.6	2.688	0.2783
ED (%, 0.05h^{-1})	59.9^{ef}	57.3^{f}	61.6^{e}	57.5^{f}	0.957	0.0270
CP						
a (%)	54.3^{f}	45.1^{g}	61.9^{e}	34^{h}	0.884	<0.0001
b (%)	36.5^{g}	51.6^{f}	32.1^{g}	59.9^{e}	1.052	<0.0001
c (h^{-1})	0.0658^{e}	0.0487^{ef}	0.0374^{f}	0.0367^{f}	0.005	0.0202
a+b (%)	90.8^{f}	96.6^{e}	94^{ef}	93.8^{ef}	1.171	0.0532
ED (%, 0.05h^{-1})	74.7^{e}	70.4^{f}	75.6^{e}	58.8^{g}	0.638	<0.0001
NDF						
a (%)	25.6^{e}	8.5^{g}	18.1^{f}	18.3^{f}	0.856	<0.0001
b (%)	59.9^{f}	83.3^{e}	61.6^{f}	61.9^{f}	4.201	0.0064
c (h^{-1})	0.0207	0.0259	0.0238	0.0200	0.003	0.5840
a+b (%)	85.5	91.8	79.7	80.3	4.795	0.3277
ED (%, 0.05h^{-1})	42.0^{e}	36.3^{f}	37.9^{ef}	35.5^{f}	1.238	0.0177

a, rapidly fraction; b, slowly degradable fraction; c, rate of degradation; ED, effective degradability = a + $[(b \times c)/(c + K_p)]$; k_p, rate of passage assumed at 0.05 h^{-1}.

[1] Dry matter.

[2] Crude protein.

[3] Neutral detergent fiber.

[4] Total mixed ration.

[5] Dry tofu cake.

[6] Rice bran.

[7] Wet green tea waste.

[8] Standard error of means.

[e, f, g] Means within rows with different letters differ ($P < 0.005$).

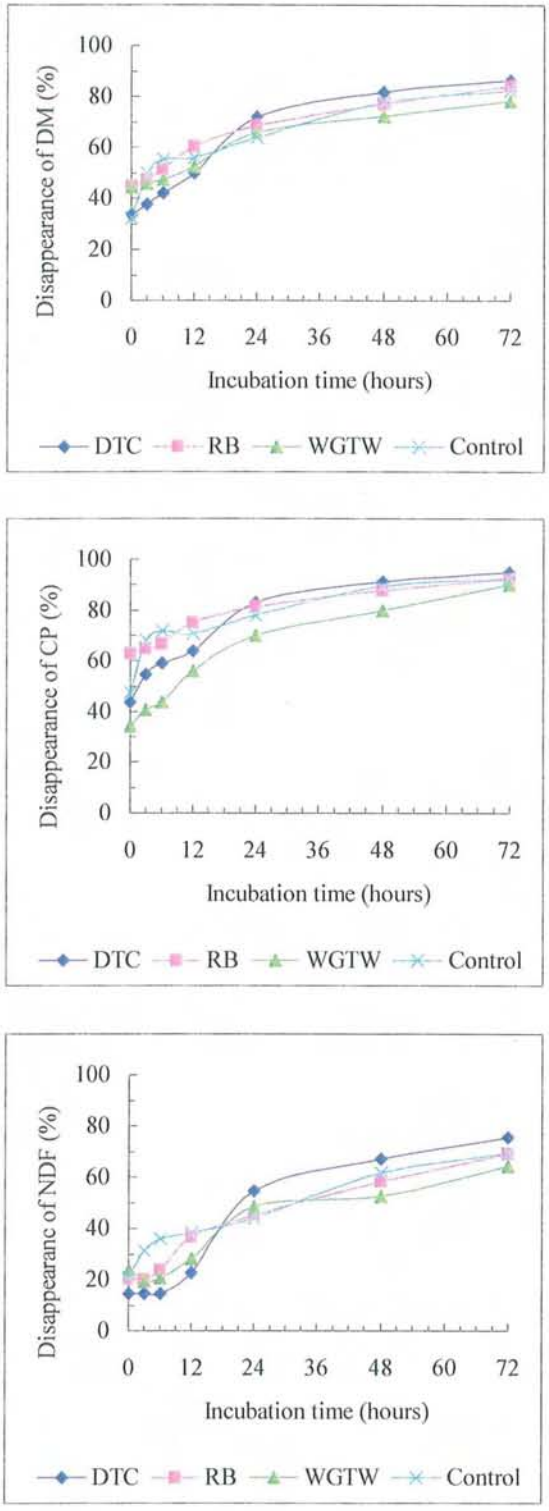

Figure 3.1. Evolution with incubation time of the dry matter (DM), crude protein (CP) and neutral dergent fiber (NDF) degradability of TMR silages. DTC, dry tofu cake; RB, rice bran; WGTW, wet green tea waste.

Table 3.6. Nutrient digestibility, nutrient content, and nitrogen retention in the TMR silage fed to wethers[1]

	Treatment				SEM[5]	P value
	Control	DTC[2]	RB[3]	WGTW[4]		
Apparent digestibility						
Dry matter (%)	72.4^{ab}	76.5^{a}	70.2^{b}	71.1^{b}	1.91	0.0121
Organic matter (% DM[6])	76.5^{ab}	79.8^{a}	74.8^{b}	74.4^{b}	1.68	0.0163
Crude protein (% DM)	63.7^{b}	76.0^{a}	66.6^{b}	73.1^{a}	2.36	0.0010
Ether extract (% DM)	70.2^{c}	82.2^{a}	76.1^{b}	63.6^{d}	1.48	<0.0001
Nitrogen free extract (% DM)	83.2^{ab}	83.7^{a}	81.2^{ab}	80.3^{b}	1.32	0.0316
Crude fiber (% DM)	54.9^{b}	67.2^{a}	54.8^{b}	57.2^{b}	2.78	0.0075
Acid detergent fiber (% DM)	58.0^{b}	69.6^{a}	60.8^{ab}	57.5^{b}	4.78	0.0103
Neutral detergent fiber (% DM)	65.8^{b}	72.0^{a}	64.2^{b}	63.1^{b}	2.51	0.0086
Gross energy (% DM)	73.1^{ab}	75.6^{a}	71.9^{ab}	69.6^{b}	1.57	0.0097
Nutrient content						
Total digestible nutrents (% DM)	72.6^{bc}	79.1^{a}	75.7^{ab}	70.8^{c}	1.62	0.0017
Digestible crude protein (% DM)	6.0^{c}	11.5^{a}	7.9^{b}	11.1^{a}	0.30	<0.0001
Digestible energy (kcal/g DM)	3.2^{bc}	3.5^{a}	3.3^{ab}	3.1^{c}	0.07	0.0017

[1]Feeding level per day: 250 g DM/kg BW.

[2]Dry tofu cake.

[3]Rice bran.

[4]Wet green tea waste.

[5]Standard error of the mean.

[6]Dry matter.

[a,b,c]Means within rows with different letters differ ($P < 0.05$).

Table 3.7. Digestibility and nutrient content of DTC, RB, and WGTW

	DTC[1]	RB[2]	WGTW[3]	SEM[4]	P value
Apparent digestibility					
Dry matter (%)	80.8[a]	67.9[ab]	63.8[b]	3.91	0.0483
Organic matter (% DM[5])	87.1[a]	70.8[b]	69.8[b]	2.90	0.0093
Crude protein (% DM)	85.1[a]	70.3[b]	79.5[ab]	2.88	0.0326
Ether extract (% DM)	88.2[a]	77.6[b]	56.2[c]	1.62	<0.0001
Nitrogen free extract (% DM)	85.6[a]	72.9[b]	70.9[b]	3.15	0.0431
Crude fiber (% DM)	95.4[a]	54.3[b]	62.9[b]	6.12	0.0106
Acid detergent fiber (% DM)	92.4[a]	70.5[ab]	56.9[b]	4.78	0.0051
Neutral detergent fiber (% DM)	89.2[a]	58.5[b]	57.4[b]	4.37	0.0022
Gross energy (% DM)	80.4[a]	69.4[b]	62.4[b]	2.39	0.0037
Nutrient content					
Total digestible nutrents (% DM)	96.9[a]	86.7[a]	73.3[b]	2.84	0.0019
Digestible crude protein (% DM)	25.6[a]	11.9[b]	25.4[a]	0.65	<0.0001
Digestible energy (kcal/g DM)	4.3[a]	3.7[a]	3.1[b]	0.13	0.0012

[1]Dry tofu cake.

[2]Rice bran.

[3]Wet green tea waste.

[4]Standard error of the mean.

[5]Dry matter.

[a,b,c]Means within rows with different letters differ ($P < 0.05$).

Table 3.8. Nitrogen retention in the TMR[1] silage fed to wethers

	Treatment				SEM[5]	P value
	Control	DTC[2]	RB[3]	WGTW[4]		
Nitrogen intake (g/day)	10.48[c]	17.36[a]	13.64[b]	17.33[a]	0.52	<0.0001
Fecal excretion of nitrogen (g/day)	3.80[b]	4.14[ab]	4.54[ab]	4.68[a]	0.35	0.0467
Urinary excretion of nitrogen (g/day)	3.00	7.44	4.47	6.19	0.76	0.0781
Nitrogen retention (g/day)	3.68	5.78	4.64	6.45	1.02	0.2658
Nitrogen retention (%)	34.84	33.2	33.5	36.56	6.06	0.9674
Allantoin (g/day)	1.13	1.74	1.45	1.87	0.20	0.2405

[1]Total mixed ration.

[2]Tofu cake.

[3]Rice bran.

[4]Green tea grounds.

[5]Standard error of means.

[a,b,c]Means within rows with different letters differ ($P < 0.05$).

Table 3.9. pH, volatile fatty acid (VFA), and ammonia-N in rumen fluid of sheep fed TMR[1] silage

	Hours after feeding	Treatment				SEM[6]	P value
		Control	DTC[3]	RB[4]	WGTW[5]		
pH	0	7.17	7.14	7.22	7.25	0.07	0.4921
	2	6.38	6.35	6.26	6.42	0.11	0.3129
	4	6.53	6.45	6.33	6.49	0.06	0.2718
Total VFA (mmol/dL)	0	4.87	4.35	3.93	4.61	0.39	0.1902
	2	8.06	6.67	7.84	8.80	0.62	0.1575
	4	7.92	8.62	8.20	9.51	0.50	0.1909
Acetic acid (A) (mol%)	0	65.10^{a}	59.07^{b}	59.08^{b}	66.28^{a}	1.12	0.0026
	2	64.54^{ab}	59.9^{b}	61.48^{ab}	67.08^{a}	2.90	0.0277
	4	64.64^{b}	62.64^{b}	62.88^{b}	70.72^{a}	3.40	0.0004
Propionic acid (P) (mol%)	0	20.61^{ab}	21.9^{ab}	25.55^{a}	19.02^{b}	1.24	0.0277
	2	23.85	26.82	27.32	24.14	2.76	0.7527
	4	21.85	25.70	26.73	19.75	1.98	0.0788
Isobutyric acid (mol%)	0	0.43^{ab}	0.46^{ab}	0.54^{a}	0.33^{b}	0.09	0.0368
	2	0.00	0.00	0.00	0.01	0.00	0.7737
	4	0.01	0.00	0.01	0.00	0.00	0.2126
Butyric acid (mol%)	0	12.70	16.64	11.29	13.37	1.17	0.0722
	2	11.26	12.8	10.89	8.25	2.05	0.5947
	4	13.24	11.42	10.1	9.29	2.19	0.5068
Isovaleric acid (mol%)	0	0.85^{b}	1.41^{ab}	2.46^{a}	0.71^{b}	0.39	0.0183
	2	0.16	0.21	0.13	0.23	0.04	0.3759
	4	0.02	0.04	0.02	0.02	0.01	0.2448
Valeric acid (mol%)	0	0.31^{b}	0.52^{ab}	1.08^{a}	0.28^{b}	0.15	0.0113
	2	0.18	0.26	0.19	0.29	0.05	0.4140
	4	0.24	0.20	0.27	0.22	0.04	0.3440
A/P[2]	0	3.21^{ab}	2.71^{ab}	2.38^{b}	3.58^{a}	0.28	0.0322
	2	2.85	2.27	2.40	2.98	0.38	0.3495
	4	3.10	2.46	2.58	3.65	0.39	0.0506
NH_3-N (mg/dL)	0	10.33^{bc}	13.45^{a}	8.99^{c}	11.55^{ab}	0.80	0.0032
	2	24.52^{b}	30.66^{a}	24.82^{b}	24.79^{b}	1.52	0.0191
	4	17.14^{c}	26.32^{a}	21.71^{b}	21.99^{ab}	1.64	0.0026

[1]Total mixed ration.

[2]Acetic acid/Propionic acid.

[3]Dry tofu cake.

[4]Rice bran.

[5]Wet green tea waste.

[6]Standard error of the mean.

[a,b,c]Means within rows with different letters differ ($P < 0.05$).

Table 3.10. Hematocrit, glucose and urea-N in blood plasma of sheep fed TMR[1] silage

	Hours after feeding	Treatment				SEM[5]	P value
		Control	DTC[2]	RB[3]	GTG[4]		
hematocrit(%)	0	31.69	31.38	30.50	32.56	1.09	0.2746
	2	31.31	30.69	30.19	29.75	1.40	0.5258
	4	29.06	28.56	28.56	30.00	1.25	0.3323
glucose(mg/dL)	0	71.71	73.21	65.82	70.00	3.91	0.5494
	2	66.08	70.79	68.83	64.77	4.39	0.6726
	4	71.18	74.06	72.23	69.09	3.90	0.6005
urea nitrogen(mg/dL)	0	11.63^{c}	18.66^{a}	13.84^{bc}	16.60^{ab}	1.00	0.0089
	2	10.60^{c}	16.6^{a}	14.03^{b}	13.00^{b}	1.07	<0.0001
	4	9.67^{c}	16.65^{a}	14.03^{ab}	12.64^{bc}	1.29	0.005

[1]Total mixed ration.

[2]Dry tofu cake.

[3]Rice bran.

[4]Wet green tea waste.

[5]Standard error of means.

[a,b,c]Means within rows with different letters differ ($P < 0.05$).

Table 3.11. The rank order of palatability from the relative intake ration of dry matter among the four TMR[1] silages fed to wethers

	Relative intake ration (DM[2])					Preference	
	Control	DTC[3]	RB[4]	WGTW[5]	Means	Rank order	Fraction
Control		0.317	0.627	0.510	0.485	2	72
DTC	0.683		0.718	0.613	0.671	1	100
RB	0.373	0.282		0.537	0.397	4	59
WGTW	0.490	0.387	0.463		0.447	3	67

[1]Total mixed ration.

[2]Dry matter.

[3]Dry tofu cake.

[4]Rice bran.

[5]Wet green tea waste.