Doctoral Dissertation (Shinshu University)

Establishment of procedures for integrative mitigation of greenhouse gases relating to ruminant production: effects of feeding fruits by-products

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反芻家畜生産に関連した温室効果ガスの複合的緩和手法の確立：果物副産物給餌の効果

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List of abbreviations

Abbr	Full description	**Abbr**	Full description
ADF	Acid detergent fiber	ND	Not detected
BP	Beet pulp	NDF	Neutal detergent fiber
BW	Body weight	NFC	Non-fiber carbohydrate
CFU	Colony forming unit	PEG	Polyethylene glycol
CONT	Control	PS	Persimmon skin
CP	Crude protein	PSA	Unfermented persimmon skin
CT	Condensed tannins	PSM	Plant secondary metabolites
DM	Dry matter	PSS	Persimmon skin silage
DMI	Dry matter intake	SD	Standard deviation
FB	Fruit by-products	SE	Standard error
FM	Fresh matter	SIL	Ensiled fruit by-products
FRE	Unfermented fruit by-products	TEPH	Total extractable phenolics
GHG	Greenhouse gas	TMR	Total mixed ration
GP	Grape pomace	VFA	Volatile fatty acid
KP	Kraft pulp	VP	Wild grape pomace
LAB	Lactic acid bacteria	WB	Wheat bran
LB	*Lactobacillus buchneri*		

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Chapter I

General Introduction

In the coming decades, the world demand for animal products will continue to increase driven by growing human populations. According to the Food and Agriculture Organization (2006), the need for meat and milk products is likely to increase to 465 million tons and 1.043 million tons, respectively. The animal industry sector has been facing contradictory challenges to produce more and to counteract climate change. The agricultural sector is responsible for a considerable amount of greenhouse gases (GHG), including carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) (Gerber et al., 2013) that accounts for 14.5% of the global anthropogenic emissions, the sum of 9 % of global CO_2, 65 % of the anthropogenic N_2O, and about 35% of CH_4. A 60% increase in the world methane production is estimated by 2030 (FAO, 2003) if methane emissions remain to increase with the livestock numbers. Enteric methane also represents a loss of energy to the animal accounting for 6-12 % of gross energy ingested by the animal (Johnson and Johnson, 1995), but the assumption depends on several factors, including feed intake, concentrate and forage ratio, and fat level. Consequently, there is a need to determine approaches to mitigate methane emissions without affecting animal and whole-farm productivity.

It is of importance for ruminant feeding to elucidate in detail how the fermentation processes are controlled in the rumen. Carbohydrates represent the largest single portion of ruminant's diets (60-70%). In the rumen, polysaccharides (mainly cellulose, hemicellulose, and starch) are degraded into monosaccharides such as glucose and other hexoses and pentoses. These monosaccharides are anaerobically broken down into pyruvate as a part of glycolysis, which are converted to VFAs and carbon dioxide with discharging a certain amount of hydrogen. The fermentation of monosaccharides to VFA associates with metabolic hydrogen removal that is used for the reduction

of the intercellular co-factors such as nicotinamide adenine dinucleotide hydride (NADH) but this co-factor must be re-oxidized to NAD to keep the fermentation process on. Through this re-oxidization process H^+ plays the role of electron acceptor from NADH and producing H_2, which used by methanogens as the electron donor to reduce carbon dioxide and generate methane. As such, the utilization of hydrogen in methane production is an essential process for the stability of rumen ecology (Russell, 2002) whereas hydrogen accumulation in the rumen inhibits microbial growth and fiber digestion (Eckard et al., 2010) (Figure1.1). Any abatement strategy that inhibits methanogen activity or decreases its numbers must offer an alternative sink to the hydrogen produced rather than methane production (McAllister and Newbold, 2008). One another pathway that can compete with methanogenesis for hydrogen is propionate production. Indeed increasing propionate production is well associated with a reduction in CH_4 generation (Janssen, 2010), and the discarded energy with methane would be retained in the body, as propionate is a glycogenic VFA (i.e., glucose precursor) in ruminants (Herdt, 1988). The increase of propionate production helps ruminants who are easily in short of molecular glucose productivity though enhancing the energy status of the ruminants, even more, decrease its methane emission (Ungerfeld, 2015; Wang et al., 2018).

Different mitigation strategies have been investigated which can be roughly classified into three main groups: 1) animal manipulation 2) diet manipulation, and 3) rumen manipulation (Cottle et al., 2011; Eckard et al., 2010), (Figure 1.2). These strategies have been applied for multiple purposes of decreasing H_2 production, stimulate H_2 to an alternative sink, and/or inhibit methanogenic archaea (number and/or activity) (Pereira et al., 2015). Among strategies with different practicability, efficiency, durability, ionophores antibiotics (monensin) have been widely recognized candidate as methane mitigants. They can inhibit methane generation by inhibiting the

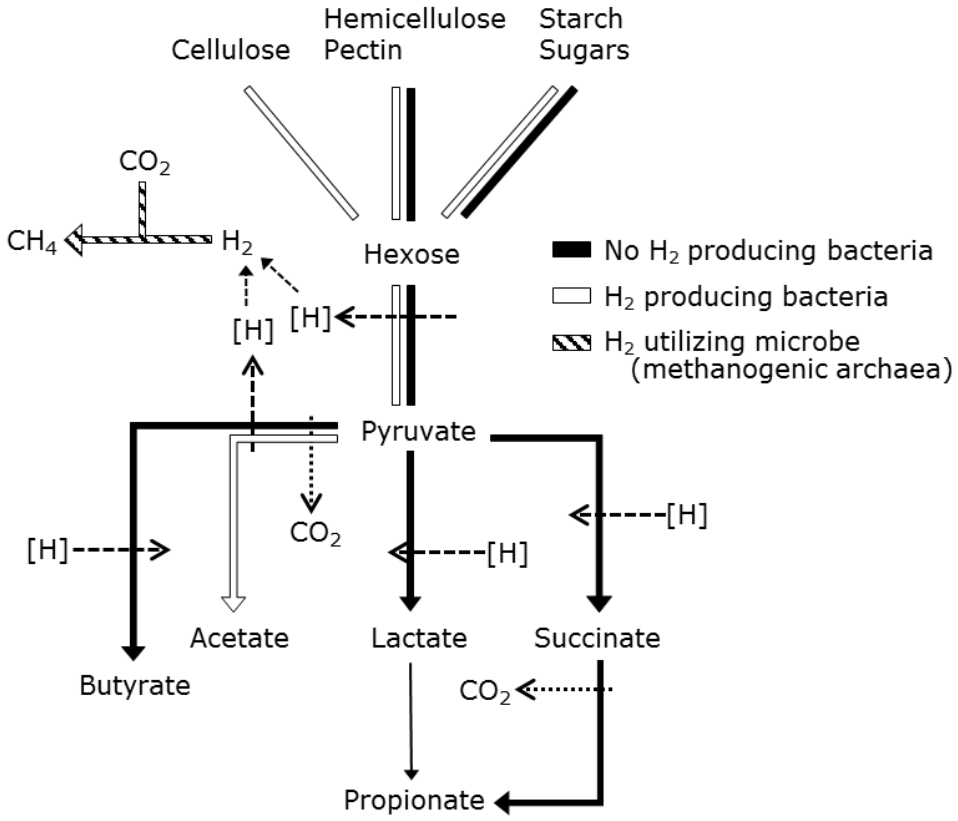


Figure 1. 1 A schematic diagram of carbohydrate fermentation characteristics of predominant ruminal bacteria which are classified into three groups with respect to H_2 production/utilization (adapted from Uyeno (2015))

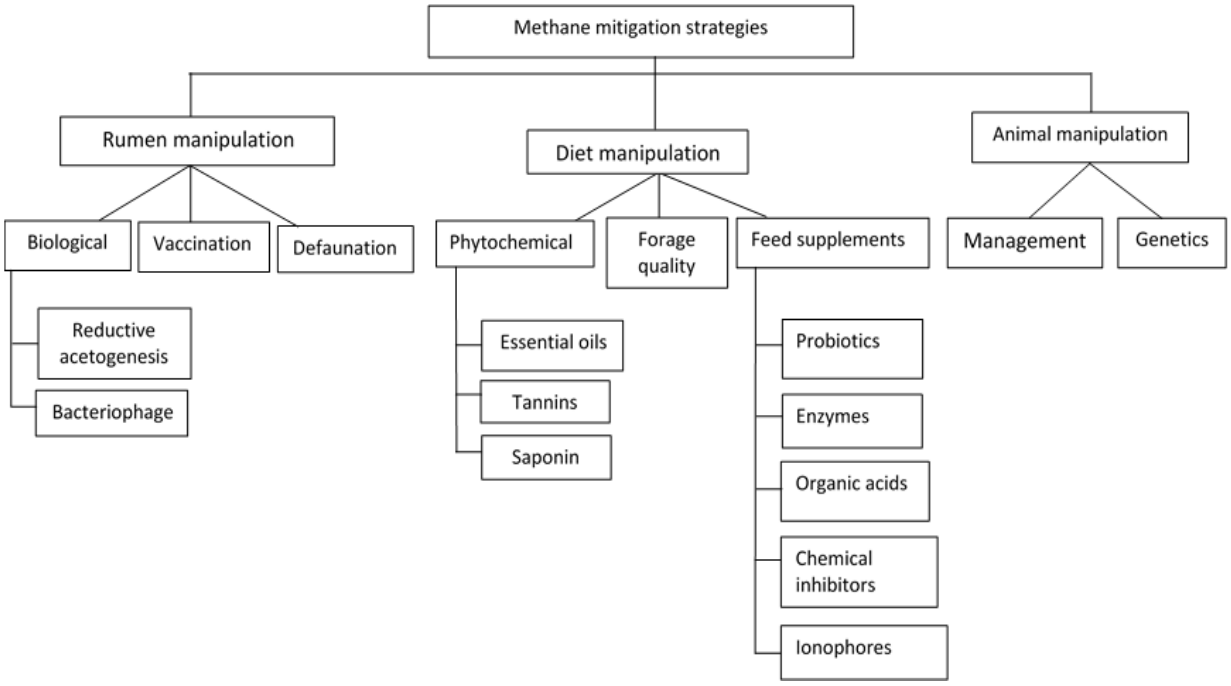
activity of hydrogen-producing bacteria, reducing the protozoal population in the rumen, increasing the propionate production, and increasing the efficacy of feed utilization. The antimethanogenic effect of monensin may be short-lived; a study in dairy cows showed that the long term administration of monensin did not affect the quantity or diversity of methanogens due to the microbial adaptation (Hook et al., 2009). Moreover, the use of antibiotics is highly associated with the health hazard because of its residues in animal products (milk and meat), which result in the rise of antibiotic-resistant bacteria. The use of antibiotics including monensin in animal feeding has

been banned in the European Union (2003). Researchers and animal nutritionists are now focusing on finding harmless and acceptable substitutes for these substances (Benchaar, 2020). Some chemical inhibitors like bromoethanesulphonic acid, an analog to methyl-coenzyme-M (co-factor involved in methanogenesis) have been shown effective in impeding methane emission by up to 50% in vivo (Hristov et al., 2013; Romero-Perez et al., 2015), which is, however, also prohibited because of its toxicity to the rumen microbiota and ozone-depleting effect (Hristov et al., 2013). Therefore, finding strategies that can decrease methane output from livestock with neither adverse effects on animal productivity and the environment nor any regulational objection is becoming a complex task.

On the other hand, dietary approaches represent the most effective strategy as it can directly affect rumen fermentation pattern and the fermentation end products. Plant secondary metabolites (PSM) are classes of the phytochemical compounds that are not primarily involved in the major biochemical processes, but they are necessary for the plant to survive in the environment (Harborne, 2001). Plant secondary metabolites have shown beneficial effects on the animal gut, such as modulating rumen fermentation profile, decreasing methane emission, and improving feeding efficiency and nitrogen utilization (Patra and Saxena, 2010).

Tannins constitute a major class of PSM and widely distributed in forage trees, shrubs, legumes, grains, vegetables, and fruits. They include water-soluble polyphenolic compounds (hydrolyzable tannin) due to its chemical structure (multiple phenolic hydroxyl groups) that has a high affinity to bind with various macromolecules, mainly proteins and, to a lesser degree, with carbohydrates and metal ions (Makkar, 2003a). The tannin binding properties protect the protein from microbial degradation, increasing the undegradable protein in the rumen (Mokhtarpour et al., 2016). The use

(a)



(b)

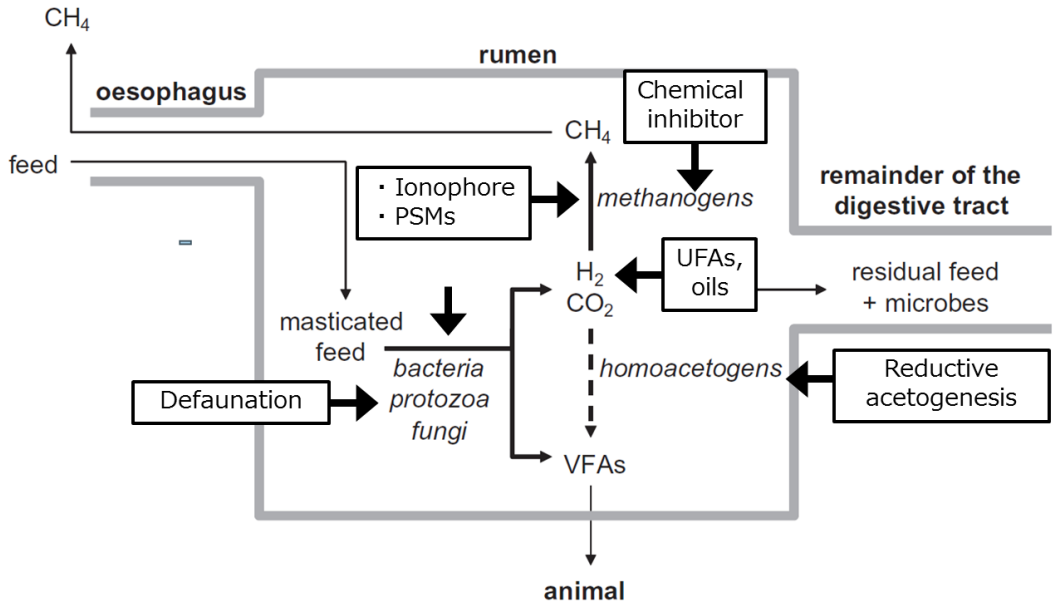


Figure 1. 2 (a) Summary of enteric methane mitigation strategies, adapted from Eckard et al. (2010), **(b)** Effectiveness of respective agents and technologies where those works for methane suppression.

of tannin-rich by-products has been described as an anti-methanogenic strategy (Goel and Makkar, 2012; Malik et al., 2017; Moate et al., 2014) by hindering methanogenesis either directly through inhibition of methanogen activity or indirectly by inhibiting protozoa and other bacterial species involved in ruminal hydrogen production (Bhatta et al., 2009; Malik et al., 2017). Profiles of two types of tannins can be summarized in the following table (Table 1.1).

Table 1.1. Profiles of two types of tannins (hydrolysable tannin and condensed tannin).

Hydrolysable tannin (HT)	Condensed tannin (CT)
Inhibition of the growth and/or activity of methanogens and/or hydrogen-producing microbes (direct effect)	Decrease methane through reduction of fiber digestion (indirect effect)
A central sugar to which a number of phenolic carboxylic acids are bound by esters of gallic acid (gallotannin) or ellagic acid (ellagitannins).	Condensation of flavan-3-ol (catechin) or flavan-3,4-diol (epigallocatechins or delpinidins) subunits linked through interflavan bonds.
Widely distributed in oak, and acacia species, especially in the browse, with a level up to 200 g HT/kg DM.	Quebracho and mimosa are CT-rich tropical plants, which sometimes have levels of 170 g CT/kg DM..

The reported antimethanogenic effect of tannin ranges widely (4.3% ~ 70% in vitro and 6.0% ~ 68% in vivo) depending on tannin type, molecular weight, amount, dietary substrate and animal species (Aboagye and Beauchemin, 2019). One negative effect of tannin on the animal is palatability issues due to its binding with oral protein resulting in an astringent sensation in the mouth (Lesschaeve and Noble, 2005) that suppresses the voluntary feed intake. Tannins may also interfere with ruminal fiber digestion by decreasing the number and/or the activity of cellulolytic bacteria (Makkar et al., 1995; McSweeney et al., 2001), which would reduce the available hydrogen for methanogenesis (Carulla et al., 2005). High levels of tannins (>5.0 g/100 g dry matter [DM])

can even be toxic or even lethal to the animal (Reed, 1995). It is essential to explore and measure the effects of feeding tannin-rich by-products on enteric methane production, taking in consideration that in addition to tannin characteristics, its effects are also highly confounded with other factors such as type, digestibility, and processing (drying, ensiling, etc) of the tannin-rich by-products itself, and the overall diet ingredients besides the animal factors.

By now, there is a significant shortage of traditional feed sources in most of the developing countries including Egypt. In Egypt, the author's home country, annually produced amount of total digestible nutrient is 9.6 million tons, which only covers 75% of the livestock energy requirements. Unavailability and the relatively high cost of feedstuff in Egypt are the major problems in animal production. To explore the locally available non-traditional feed resources such as food by-products (FB) is one feasible approach to fill the gap and to decrease feeding costs. Fruits by-products represent a large proportion of the food wastes with the global production of fruits was estimated by more than 865 million metric tons in 2017 (FAO, 2017). The by-products account for about 30% on average (Kasapidou et al., 2015). Along with the fact that FBs are rich sources of various nutrients like sugar, mineral, and digestible fiber, they are also a rich source of valuable PSM in particular tannin.

Grape pomace (GP) is the by-product that resulted from industrial processing and composed mainly of the skin, stem, and seed. During the juice production, about 25% of the total grape weight is wasted (Hassan et al., 2019). The chemical composition of GP is a valuable option for ruminant supplementation during the forage shortage (Massaro Junior et al., 2020). Grape pomace has been known as possible source of bioactive compounds, mainly polyphenols; it has already been used for the extraction of anthocyanins (Bordiga, 2016). Recently supplementing lamb diets with sun-dried GP with different levels (5-20%) for 42 days did not affect the carcass traits (Chikwanha et

al., 2019). Comparable outcomes were also stated by others, with positive effects on fecal microbiota, meat quality, and meat fatty acid profile of the involved lambs (Kafantaris et al., 2017; Kafantaris et al., 2018). A study on Holstein-Friesian calves fed GP and copper sulfate for 75 days showed an alteration in rumen microbial profile in terms of both diversity and function of the rumen microbiota (Biscarini et al., 2018). In addition to GP high tannin content, it contains a considerable amount of fat, lignin, and tartaric acid, which all have the potential to suppress methane emission (Moate et al., 2016). The high proportion of GP resulted in reduced DM intake, rumen digestibility, and negatively affected nitrogen retention in sheep (Abarghuei et al., 2010; Nudda et al., 2015). An elucidation for these adverse effects can be found in grape composition which is the high content of the phenolic compound e.g., tannins (Lu and Yeap Foo, 1999), which considered one of the probable reasons for its positive effects.

Persimmon skin (*Diospyros kaki*, PS) is a widely-distributed fruit in Asian countries. China, Korea, and Japan are among the leading countries producing such fruit. According to the FAO statistics in 2017, the production of these countries from persimmon fruits was respectively 4.0, 0.4, and 0.2 million tons, which represent more than 75% of global production. Persimmon fruit has two types, sweet and astringent, the latter usually used for dried persimmon production (*Hoshigaki* in Japanese). The drying process is done by peeling the fruit, which results in a significant amount of PS. The dried persimmon is prevalent in Nagano prefecture; most of the harvested fruits used for this industry generating a significant amount of skin estimated about 2,500 tons/ year. This skin is rich in soluble crude fiber such as pectin as well as soluble carbohydrates. Persimmon skin has a considerable level of PSM like phenolic compounds, particularly tannin (Lee et al., 2008; Yaqub et al., 2016). The utilization of PS as a livestock feed is limited due to its bulky nature and short shelf life because of the high soluble carbohydrate, which makes it ready for rapid spoilage.

The seasonal production, variable bioactive compounds, and high soluble carbohydrate content are the main constraints militating against the usage of FBs as a sustainable feed source. The ensiling of by-products seems an appropriate method to overcome these obstacles. This method can enhance palatability, significantly reduces toxic substances present in ensiled material (Moran, 2005). In addition to the chemical composition changes that occur during the ensiling, it has been reported that the ensiling of tannin-rich material might involve quantitative and qualitative changes in the tannins (Alipour and Rouzbehan, 2007; Kim et al., 2006). Researches suggested the ensiling as an economical and effective way to decrease the concentration of tannins (Alipour and Rouzbehan, 2007; Ben Salem et al., 2005; Makkar, 2003a). However, studies investigating the antimethanogenic efficiency of the ensiled tannin-rich FBs are limited, hence it could be interesting to explore to what extent the ensiling of these FBs would modify the methane generation and what is the mechanism behind it?

Regardless of the merits of the ensiling of FB, there is still a necessity to improve the technology to be introduced to agricultural waste products that are rich in soluble carbohydrate and have high moisture content. The high-moisture silages are more susceptible to draining effluent from the silo containing soluble DM losses, it is further considered as one of the watercourses pollutants (Woolford, 1978). The best way to control effluent leakage is to adjust DM content prior to ensiling. Besides, absorbents also support the nutritional enhancement to the ensiled material. Different dry absorbents have evaluated for their potential to diminish silage effluent such as brans, wheat straw, dried beet pulp, and cereal grains (Alejandro, 1991; Barmaki et al., 2018; Kordi and Naserian, 2012). There is no data about the possibility of using kraft pulp (KP) as a silage absorbent, although it has been evaluated as feed material (Maeda et al., 2019; Nishimura et al., 2019), particularly suitable for initiative solid feed for calves (Kido et al., 2019). The use of KP as an

absorbent would help optimize carbohydrate balance within FB silage in addition to minimizing the effluent production.

Objective and Study Structure

The first hypothesis from the general introduction was that the application of FBs that are rich in tannin might be beneficial in view of multiple aspects such as providing cheap feed resources, modifying the rumen fermentation profile, and decreasing methane generation. The application would be useful since the disposal of the by-products as it cannot be discarded away without stressful process, expensive cost nor sometimes leading environmental problems as they decompose in landfills and produce N_2O, which has a much higher global warming potential than either CH_4 or CO_2. In relation to this, FBs which contain a considerable level of PSM can result in changes in the rumen fermentation profile and rumen microbiota. Therefore the current research work aimed to investigate the possibility of inclusion of some available FBs including grape pomace (GP) and persimmon skin (PS) in ruminant feed, optimize the dose of each FBs which has no negative effect on the animal, and reveal their potential effect on the rumen fermentation and methane mitigation. The second hypothesis was that the ensiling would overcome the main obstacles in usage FBs, modify its chemical composition and tannin content, and consequently methane generation. In addition to that, adjusting DM content in PSS by using dry absorbent would limit the effluent leakage and enhance the nutritional value of the ensiled material.

The overall focus in this thesis was therefore to evaluate the effect of different FBs silages on the rumen fermentation and methane emission with emphasis on enhancing the PSS fermentative and nutritive quality. Therefore the following experiments were done in order: (1) to evaluate the in vitro fermentation of some ensiled FBs using the batch culture technique, (2) to reveal the

antimethanogenic activity of ensiled FBs, (3) to improve the fermentation quality of FBs silages, (4) to diminish the effluent output during the ensiling process by evaluating different absorbents, and (5) to optimize the PSS amount, which can be used in animal feed without adverse effects on feed intake and palatability.

The first study (Chapter II) was conducted to assess the impact of the incorporation of ensiled FBs on rumen microbiota and in methane emission by an in vitro study. With an emphasis on enhancing the applicability of PSS, the second study (Chapter III) consisted of three experiments aiming at elucidation of the effect of dry absorbents on PSS quality, in vitro rumen fermentation kinetics, and ingestive behavior by animal.

Chapter II

Evaluation of in vitro ruminal fermentation of ensiled fruits by-products and their potential for feed use

Abstract

Ensiling of tannin-rich FBs involves quantitative and qualitative changes in the tannins, which would consequently change the rumen fermentation characteristics. This study aimed to evaluate whether ensiled FBs are effective in mitigating methane emission from ruminants by conducting in vitro assessments. Fruits by-products (grape pomace, wild grape pomace, and persimmon skin) were collected and subjected to four-week ensiling by *Lactobacillus buchneri (L. buchneri)* inoculant. A defined feed component with or without FB samples (both fresh and ensiled material) was subjected to in vitro anaerobic culturing using rumen fluid sampled from beef cattle, and the fermentation parameters and microbial populations were monitored. Reduced methane production and a proportional change in total volatile fatty acids (especially enhanced propionate proportion) were noted in bottles containing the FBs compared with that in control (without FB). In addition, lower gene copy number of archaeal 16S rRNA and considerably higher levels of one of the major fibrolytic bacteria (*Fibrobacter succinogenes*) were detected in the bottles containing FBs than in control, particularly, when it was included in a forage-based feed. However, in the following cultivation experiment, FBs failed to exhibit a significant difference in methane production with or without polyethylene glycol, implying that tannins in the FBs may not be responsible for the mitigation of methane generation. The results of the in vitro cultivation experiments indicated that not only the composition but also the ensiling of FBs affected rumen fermentation patterns and the degree of methane generation. This is primarily because of the compositional changes in the fibrous

fraction during ensiling as well as the presence of readily fermented substrates, whereas tannins in these FBs seemed to have little effect on the ruminal fermentation kinetics.

Introduction

The potential benefits of feeding GP and PS which contain certain amounts of tannin might include manipulation of the rumen microbial community to reduce methane eructation and improve feeding efficiency. The Fermentation quality and aerobic stability of GP and PS silage have been evaluated and improved by using *L.buchneri* inoculum (Uyeno et al., 2016b). However, ensiling might involve quantitative and qualitative changes in the tannins content (Alipour and Rouzbehan, 2007; Kim et al., 2006) which would consequently change the rumen fermentation characteristics when the silage is fed to ruminants. Therefore, this study aimed to evaluate the silage quality of the three FBs including GP, wild grape pomace (*Vitis coignetiae*, VP), and PS. Furthermore, to reveal whether the ensiled form of these tannin-rich FBs is effective in mitigating methane emission from ruminants by conducting in vitro assessments.

Materials and Methods

Preparation of fruits by-product silages

Three types of FBs (GP, VP, and PS) were obtained from different food processing factories in Nagano Prefecture, Japan. Grape pomaces were collected from different breweries after the juicing of *Vitis labrusca* (Niagara, as GP) and *Vitis coignetiae* (as VP). Persimmon skin was collected from a processing plant for dried persimmon. Respective FB samples were collected from each factory twice during each harvesting season in 2016, and all the FB samples were frozen immediately after collection until use. After thawing, two batches of equal amounts of each FB sample were mixed well, and proximate composition in the mixture was analyzed according to the official methods of AOAC (AOAC, 1990). The total extractable phenolics and condensed tannins (CTs) were determined using the method of Makkar (2003b). The CT contents in the FBs were quantified as

leucocyanidin equivalents, as reported in previous studies (Makkar, 2003b). The nutritional values of each FB are shown in (Table 2.1). Before ensiling, the water content of the PS samples was adjusted by the addition of corn cobs to 95 g/kg DM PS. Following +previous studies, *L. buchneri* NBRC107764 was used for fermentation in this study (Hiramori et al., 2015; Uyeno et al., 2016b). The stock culture of this strain was used for the inoculation of FB (1% v/w). The inoculated material was mixed well by hand, and 50 g of each was packed in a three-layer film bag. The bags were vacuum packed and tightly heat sealed (SQ-205S; Asahikasei Packs Co. Ltd., Tokyo, Japan), and then incubated at 25°C. Ensiled content was diluted by the addition of 450 mL of saline to the bags and incubated for 2 h at 5°C. The diluted sample was used to determine the pH, LAB counts (by using MRS agar [Oxoid, Basingstoke, UK]), yeast counts (by using chloramphenicol-added potato dextrose agar), and organic acids (by using HPLC as described previously (Hiramori et al., 2015). The FBs ensiled for 28 days were then screened for their rumen fermentation modulation ability by using the in vitro culture method. All samples were air-dried at 60°C, ground, passed through a 1 mm screen, and stored at –20°C until they were used for the in vitro culture test.

In vitro fermentation test

Animal handling was performed according to the Shinshu University guidelines. The method of incubation was the same as that used in a previous study (Uyeno et al., 2016a). Rumen fluid samples were collected from Japanese Black beef cattle (8 months old, BW 280 kg) via a rumen fistula immediately before the morning feeding. Cattle were fed Italian ryegrass straw and commercial concentrate at a 1:1 ratio, up to 3 kg of the concentrate. The collected rumen fluid was strained through four layers of cheesecloth and diluted (1:2) with pre-warmed McDougall buffer, which had been flushed with CO_2 gas, and then used within 2 h after collection. The diluted rumen fluid (40

mL) was dispensed into a 100 mL serum bottle containing substrate (1.0 g) and then was flushed again with CO_2 gas. Two consecutive in vitro experiments were conducted as follows: Experiment 1. Six treatments have been evaluated, Two types of a basal feed mixture were used (concentrate-based and forage-based, including 80% of a designated part and 20% of the remaining part), each was mixed with fresh FBs that were maintained at –20°C (FRE), ensiled FB (SIL), or the basal feed (CONT) to one-third of total DM. The concentrate was a commercial product, and the forage was dried Italian ryegrass (Table 2.1). Depending on the results from experiment 1, Experiment 2, was conducted only using a forage-based feed that mixed with FRE, SIL, or CONT to one-third of total DM; subsequently, each feed mixture was subdivided into two with or without polyethylene glycol (PEG; 200 mg/g feed; PEG-6000; Wako Pure Chemical Industries Ltd., Osaka, Japan), accounting to six experimental substrates. The PEG was added to determine the effects of tannin on in vitro methanogenesis(Abarghuei et al., 2010; Bhatta et al., 2009). Because of material availability, Experiment 2 was performed using only GP and PS samples. The serum bottles (n = 3 per group) were sealed with a butyl rubber stopper and aluminum cap, and then incubated anaerobically for 24 h at 39°C with shaking at 180 rpm in a water bath.

Sample analysis

After incubation, fermentation parameters (headspace gas composition, organic acid content, and methane generation) were analyzed according to the methods described previously (Abrar et al., 2016; Denman et al., 2007). The total bacterial numbers, methanogens, and fibrolytic bacteria were quantified using a real-time polymerase chain reaction method. Genomic DNA of the microorganisms was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and the nucleic acid material was stored at less than –

20°C until analysis. The polymerase chain reaction (PCR) conditions and primer sequences for total bacteria, Archaea, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens* were following previous literature (Lettat et al., 2013; Stevenson and Weimer, 2007). Primer sets for the real-time PCR were Eub338F (ACTCCTACGGGAGGCAG) and Eub522R (ACGTCRTCCMCNCCTTCCTC) for total bacteria, qmcrA-F (TTCGGTGGATCDCARAGRGC) and qmcrA-R (GBAR GTCGWAWCCGTAGAATCC) for Archaea, Fsuc3F (GTTC GGAATTACTGGGCGTAAA) and Fsuc3R (CCCCCGGAC ACCCAGTAT) for *F. succinogenes*, and RumFla3F (TGGCGG ACGGGTGAGTAA) and RumFla3R (TTACCATCCGTTTC CAGAAGCT) for *R. flavefaciens*. CFX96 Real-Time system (Bio-Rad Inc., Hercules, CA, USA) and a SYBR(R) Premix Ex Taq Kit (Takara Bio Inc., Otsu, Japan) were applied for the real-time PCR. The cycling conditions were initial denaturation at 95°C for 10 s, and 40 cycles at 95°C for 5 s, 62°C for 30 s, followed by melting curve analysis to confirm that expected PCR products were obtained.

Statistical treatment

Analysis of variance (ANOVA) was applied for each measurement in each experiment. The following model was used:

$Y_{ijk} = \mu+\alpha_i+\beta_j+\gamma_k+(\alpha\beta)_{ij}+(\beta\gamma)_{jk}+(\gamma\alpha)_{ki}+(\alpha\beta\gamma)_{ijk}+e_{ijk}$

Where Y_{ijk} = observations for dependent variables; μ = overall mean; α_i = the fixed effect of FB material (GP, VP, PS); β_j = the fixed effect of replacement (CONT, FRE, SIL); γ_k = the fixed effect of base-feed (CONC, FOR; Experiment 1) or PEG inclusion (Experiment 2); $(\alpha\beta)_{ij}$, $(\beta\gamma)_{jk}$, $(\gamma\alpha)_{ki}$, $(\alpha\beta\gamma)_{ijk}$ = the interaction effect; and eijk = the residual error. Before ANOVA, the model assumptions were subjected to both a robust test for equality of variances and Shapiro–Wilk's test

for normality of residual data to check the validity of the model. A value of $p < 0.05$ on least-squares means was considered to indicate a significant effect of the treatment. Tukey's pairwise comparison was applied for the post-hoc test. All statistical analyses were performed with Stata 13.1 (Stata Corp, College Station, TX, USA).

Table 2.1. Proximate composition of fruits by-products (fresh matter) and feeds used for in vitro cultivation experiments.

Items	GP	VP	PS	Concentrate	Italian ryegrass
DM (g/kg)	335	445	251	931	905
CP (g/kg DM)	95	145	45	180	95
NDF (g/kg DM)	250	340	220	220	680
NFC (g/kg DM)	495	448	680	405	85
TEPH (g/kg DM)	113	103	210	NA	NA
CT (g/kg DM)	71	59	85	NA	NA

Abbreviations, GP, grape pomace; VP, wild grape pomace; PS, persimmon skin; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; NFC, non-fiber carbohydrates; TEPH, total extractable phenolics; NA, not available; CT, condensed tannins.

Results and Discussion

Quality of ensiled FBs

Developing tannin-rich FBs for feed use requires appropriate management to obtain high-quality silage without causing nutritional losses by aerobic deterioration. The fermentation data of the FBs are shown in (Table 2.2). After 28 days of fermentation, the addition of LAB did not further decrease the pH compared to that in control in GP and VP, and only lactate and acetate, but not propionate or butyrate, were detected in all the samples. However, the addition of LAB contributed to increased lactic acid fermentation in PS silage. *L. buchneri* is known to be effective in preventing aerobic deterioration when applied as an inoculant for ensiling plant feeds (Nishino et al., 2004; Tabacco et al., 2011), and its use in silage preparation contributes to DM recovery (Goeser et al., 2015). Uyeno et al. (2016b) reported that the GP and PS ensiled with *L. buchneri* showed no temperature increase under anaerobic condition, indicating positive (i.e., preservative) effect in these silages. Therefore, LAB inoculation of VP silage may have a positive effect on its storage stability even though yeast would survive in it. Indeed, no aerobic deterioration was observed for more than 10 days after opening the LAB-included VP silage, whereas slight (approximately 1°C) temperature increases were found in the raw material and control silage (data not shown).

Effects of replacement with FBs on the in vitro rumen fermentation

In Experiment 1, an in vitro assessment has been conducted to evaluate whether ensiled or non-ensiled FBs could be effective in modulating rumen fermentation as well as changing methanogen proportion (Table 2.3). As revealed by the volatile fatty acid (VFA) proportion, supplying any kind of FBs increased gas production and changed the fermentation direction to propionate production as a hydrogen sink from the generation of methane. Moreover, total VFA was likely increased with

Table 2.2. Profiles of fruits by-products and its silages tested in in vitro experiments.

Item	GP		VP		PS	
	FRE	SIL	FRE	SIL	FRE	SIL
pH	3.86	3.89	3.55	3.46	6.04	3.54
LAB (log CFU/g)	5.15	6.86	5.75	6.21	5.69	7.86
Yeast (log CFU/g)	7.49	6.02	6.11	6.12	6.81	1.80
Lactate (g/kg FM)	15.0	14.7	14.7	21.7	6.3	38.7
Acetate (g/kg FM)	2.9	3.0	8.5	9.6	2.9	16.9

Abbreviations: LAB, lactic acid bacteria; CFU, colony forming units; GP, grape pomace; VP, wild grape pomace.

the FB supplementation. However, according to post-estimation results of the interaction between fruits and base feed (Table 2.4, Table 2.5), the effect was only significant when PS was applied, presumably because of the higher soluble sugar concentration in PS. Interestingly, concurrent results were obtained in the increase of gas production and the decrease of acetate proportion in response to the addition of either type of FB (fresh material or ensiled one) to concentrate-based feed. In contrast, these results were marginally observed only with the addition of fresh material to forage-based feed. An increase in gas production (i.e., fermentation intensity) in response to the addition of fresh material is probably due to fermentable sugars in the FB. On the other hand, there are maybe different microbes responsible for FB fermentation according to its status (fresh material or ensiled one) since different results were obtained with respect to the base feed. Methane production was particularly decreased in response to FB supplementation when it was added to the forage-based feed. Interestingly, the proportion of archaea to total bacteria was the lowest in SIL, and significantly lower in FRE than in CONT. This was due to the increase in total bacterial number in SIL within forage-based feed rather than decreasing the archaeal population. As compared with

fresh FBs, the ensiled ones showed no effect on other measurements, except for a marginal difference in NH_3-N.

The hypothesis was that the effects of replacement of feed with ensiled FBs on the in vitro rumen fermentation patterns were also probably because of the changes in the form of tannins during fermentation. Fruits by-products generally include non-edible parts of agricultural products, which often contain phenolic compounds such as tannins. The extractable phenolic compounds were marginally higher in PS than in GP, whereas CT levels were similar between the two FBs (Table 2.6). This suggested that phenolic compounds other than CT, such as hydrolyzable tannin, were higher in PS than in GP, consistent with the findings of a previous study (Kondo et al., 2015). Antimethanogenic activities of tannins have been extensively revealed in several in vitro and in vivo studies (Malik et al., 2017; Patra and Saxena, 2011), although not completely. Bhatta et al. (2009) evaluated the effects of six commercially available natural sources of tannins on the total archaea by conducting in vitro culture experiments and found that CTs reduced methane production by 5.5% and suppressed the population of methanogenic archaea by 12.0%. The total archaeal population was lower when the combination of two types (hydrolyzed and CTs) was used than when hydrolyzed tannins were used alone, which might be attributed to the different modes of action of these kinds of tannins.

In addition to tannin, the characteristics of carbohydrate composition in these FBs might also primarily determine the fermentation patterns of the in vitro rumen culture. Compared to other plant materials that are rich in tannin, the inclusion of highly digestible carbohydrates with a certain amount may be a particular nature of FBs. The NFC can be immediately digested by the major members of the rumen flora (e.g., *Bacteroidetes* and *Firmicutes*) (Dehority, 2003a), and anaerobic conversion into organic acids such as succinate, propionate, and butyrate can function as alternative

hydrogen sink to methane. Theoretically, since carbohydrates available for anaerobic digestion in the in vitro culture in ensiled FBs included a higher amount of fiber than that in fresh FBs, intensive fiber digestion by fiber-degrading bacteria might occur resulting in the increased production of acetate accompanied with hydrogen, which was used for the reduction of CO_2 to methane generation. However, the amount of methane production was higher in FRE than in SIL. Conversely, as shown recently, digestibility can be improved by ensiling total mixed ration (TMR) in an in vivo digestibility assessment (Cao et al., 2012, 2010a), which was possibly associated with enhanced fiber digestion in the rumen. The ensiled TMR resulted in more methane production under in vitro rumen cultivation than fresh (non-ensiled) TMR (Chao et al., 2016). Thus, the ensiling of FBs might induce changes in the fiber composition, which would, in turn, offer favorable conditions for the growth of these fibrolytic bacteria.

Therefore, the second in vitro cultivation experiment has been performed to evaluate in detail the relationship between the compositional changes in FBs during ensiling and the rumen fermentation characteristics and microbial profiles involved in fermentation (Experiment 2). In this experiment, a forage-based feed component was chosen for the testing because it exhibited a more prominent reduction than did the concentrate-based one in methane generation in response to FB addition. In accordance with the previous experiment, in Experiment 2, a marginal decrease in methane production was observed between the FRE and SIL groups (Table 2.7). Inclusion of ensiled FBs (SIL) changed the carbohydrate profiles of the test feeds during cultivation experiments compared to those in the control (CONT, no FB addition) and even to those of FRE (Table 2.6). In the SIL group, the fraction of non-fiber carbohydrates (NFC) was marginally lower than that in FRE. This finding suggested that bacteria contributing to silage fermentation (e.g., *Lactobacillus* species) utilized these readily available carbohydrates to convert to lactate and

acetate. In contrast, with the inclusion of NDF, overall carbohydrate composition was different between GP and PS. These compositional changes seemed to certainly affect the microbial proportions during in vitro cultivation, especially for the two representative fibrolytic bacteria.

The partially degraded fiber generated from ensiling might provide more suitable fermentation substrates for *Fibrobacter* than for *Ruminococcus*, and the difference in the bacteria that participated in fiber degradation might have resulted in switching the fermentation product to limited hydrogen generation, which was expected to reduce methane production. This idea is partly supported by results obtained from experiment 2 because the absolute number of *Fibrobacter* was higher in the PS group than in the GP group. However, determining fermentation kinetics in the rumen is seemingly insufficient, owing to the monitoring of limited microbiota. A comprehensive assessment of the microbiota with the pyrosequencing approach will be helpful to understand what is changing in the rumen in response to the intervention.

The results of experiment 2 implied that mitigation of methane production could be partly attributed to tannins. However, because that two kinds of FBs failed to exhibit a significant difference in any parameter with or without PEG, except for total gas production and the interaction of the replacement and PEG inclusion on acetate proportion, the addition of PEG may have alleviated the adverse proportion changes in FRE and SIL as compared with CONT. This observation might suggest that tannins in FBs may have a considerable effect on changes in fermentation patterns in the rumen owing to some minor modifications of rumen microbe composition.

Table 2.3. In vitro rumen fermentation characteristics of concentrate-based or forage-based rations containing ensiled fruits by-products.

Item[1)]	Fruit			Base feed		Replacement			SE	Contrasts						
													interaction			
	GP (18)	VP (18)	PS (18)	Concent-rate (27)	Forage (27)	CONT (18)	FRE (18)	SIL (18)		Fruit (F)	Base feed (B)	Repla-cement (R)	F×B	F×R	B×R	F×B×R
Gas production (mL)	24.5	24.3	25.6	29.4	20.2	21.5	27.0	25.9	0.8	0.12	< 0.01	< 0.01	0.06	0.39	< 0.01	0.07
Lactate (mmol/L)	1.3	1.2	0.7	0.9	1.1	1.2	0.9	0.9	0.1	0.08	0.45	0.22	0.25	0.35	0.40	0.24
Total VFA (mmol/L)	85.3	84.2	89.9	97.6	75.3	80.6^{a}	93.4^{b}	85.4^{ab}	1.9	0.02	< 0.01	< 0.01	0.02	0.06	0.26	0.17
Acetate (mol %)	59.3	59.7	61.0	56.9	63.1	63.9	58.8	57.3	0.7	0.25	< 0.01	< 0.01	0.46	0.28	0.03	0.79
Propionate (mol %)	30.1	30.1	30.4	32.8	27.6	27.8^{a}	31.2^{b}	31.7^{b}	0.6	0.93	< 0.01	< 0.01	0.37	0.67	0.15	0.96
Butyrate (mol %)	10.6	10.2	8.6	10.3	9.3	8.3^{a}	10.0^{ab}	11.1^{b}	0.4	0.06	0.18	0.01	0.37	0.19	0.11	0.24
NH_3-N (mmol/L)	38.6	39.4	38.0	39.8	37.5	38.0	39.6	38.4	0.3	< 0.01	< 0.01	< 0.01	< 0.01	0.60	< 0.01	0.20
Methane (mmol/L)	12.6	13.0	13.1	11.9	13.8	14.9	12.6	11.0	0.3	0.30	< 0.01	< 0.01	0.15	0.51	< 0.01	0.16
Total bacteria (log10 copies/mL)	8.0	8.0	8.0	8.0	8.0	7.9	7.9	8.1	0.0	0.31	0.46	< 0.01	0.19	0.27	< 0.01	0.40
Archaea (log10 copies/mL)	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.1	0.0	0.98	0.19	0.01	0.82	0.65	< 0.01	0.12
Archaea (% total bacteria	1.5	1.7	1.9	1.6	1.7	2.2^{a}	1.8^{b}	1.1^{c}	0.1	0.10	0.23	< 0.01	0.40	0.35	0.06	0.09

Abbreviations: VFA, volatile fatty acids; GP, grape pomace; VP, wild grape pomace; PS, persimmon skin, FRE, fresh material; SIL, silage. [abc] Values with different superscripts within the same row mean significant difference. When the interaction was significant, Tukey's pairwise comparison was applied for each combination as a post-estimation. Results of pairwise comparisons were shown in Table 2.4 and Table 2.5. [1)] Numbers in parenthesis indicate a number of cultivation bottles in the experiment.

Table 2.4. Pairwise comparisons of marginal linear predictions as post-estimation of data in Experiment 1 (after ANOVA, regarding the interaction between fruits and base feed).

	Total VFA production	NH_3-N
GC vs GF	**	*
GC vs VC		
GC vs VF	**	
GC vs PC	*	
GC vs PF	**	**
GF vs VC	**	*
GF vs VF		
GF vs PC	**	*
GF vs PF		*
VC vs VF	**	
VC vs PC	*	
VC vs PF	**	**
VF vs PC	**	
VF vs PF		**
PC vs PF	*	**

Abbreviations: GC, grape pomace and concentrate; GF, grape pomace and forage; VC, wild grape pomace and concentrate; GF, wild grape pomace and forage; PC, persimmon skin and concentrate; PF, persimmon skin and forage.

Table 2.5. Pairwise comparisons of marginal linear predictions as post-estimation of data in Experiment 1 (after ANOVA, regarding the interaction between base feed and replacement).

	Gas production	Acetate	NH_3-N	Methane	Total bacteria	Archaea
CC vs CF	**	*	*			**
CC vs CS	**	**		**		**
CC vs FC	**	*		**	**	**
CC vs FF	*					*
CC vs FS	*		**	*		*
CF vs CS			*	*		
CF vs FC	**	**		**		
CF vs FF	**	*	*			
CF vs FS	**	*	*		**	
CS vs FC	**	**		**	**	
CS vs FF	**	**		**		
CS vs FS	**	**	**			
FC vs FF	*	*		**		
FC vs FS			**	**	**	
FF vs FS			**	*	**	

Abbreviations: CC, concentrate and control; CF, concentrate and fresh material; CS, concentrate and silage; FC, forage, and control; FF, forage, and fresh material; FS, forage and silage.

Table 2.6. Proximate composition of test feeds containing ensiled fruits by-products in Experiment 2.

Fruit Replacement	CONT	GP		PS	
		FRE	SIL	FRE	SIL
DM (g/kg)	925	918	916	908	903
CP (g/kg DM)	120	118	115	105	106
NDF (g/kg DM)	550	567	553	441	433
NFC (g/kg DM)	145	180	164	313	272
TEPH (g/kg DM)	10	55	35	76	71
CT (g/kg DM)	2	26	30	29	30

Abbreviations: GP, grape pomace; PS, persimmon skin, CP, crude protein; NDF, neutral detergent fiber; NFC, non-fiber carbohydrates; TEPH, total extractable phenolics; CT, condensed tannins. CONT means no inclusion of FB, FRE and SIL means inclusion of corresponding materials of each FB

Table 2.7. In vitro rumen fermentation characteristics of test feed containing ensiled fruits by-products and PEG.

Item[1)]	Fruit		Replacement			PEG		SE	Contrast			Interaction			
	GP (18)	PS (18)	CONT (12)	FRE (12)	SIL (12)	-PEG (18)	+PEG (18)		Fruit (F)	Repla-cement (R)	PEG (P)	F×R	F×P	R×P	F×R×P
Gas production (mL)	22.6	23.0	23.4	22.2	22.8	21.8	23.8	0.4	0.51	0.36	< 0.01	0.99	0.47	0.24	0.14
Total VFA (mmol/L)	92.8	95.8	97.9	94.0	91.0	93.9	94.7	1.2	0.25	0.11	0.76	0.92	0.97	0.38	0.93
Acetate (mol %)	52.8	50.5	55.3	50.5	49.2	51.3	52.0	0.9	0.11	< 0.01	0.21	0.50	0.93	<0.01[2)]	0.55
Propionate (mol %)	34.6	35.0	33.3	35.5	35.6	34.9	34.7	0.5	0.83	0.16	0.73	0.38	0.70	0.07	0.57
Butyrate (mol %)	12.6	14.5	11.4[a]	14.1[b]	15.3[b]	13.8	13.3	0.6	0.07	0.01	0.18	0.80	0.81	0.07	0.76
NH_3-N (mmol/L)	38.6	40.6	39.9	37.5	41.3	39.5	39.6	0.6	0.13	0.06	0.91	0.69	0.51	0.28	0.88
Methane (mmol/L)	13.1	13.8	15.1[a]	13.2[b]	12.1[c]	13.6	13.4	0.2	0.14	< 0.01	0.32	0.84	0.13	0.57	0.62
Total bacteria (log10 copies/mL)	8.1	8.1	8.0	8.1	8.2	8.1	8.2	0.0	0.94	0.08	0.52	0.77	0.83	0.42	0.87
Archaea (log10 copies/mL)	6.2	6.1	6.2	6.1	6.1	6.1	6.2	0.0	0.73	0.33	0.20	0.68	0.95	0.99	0.91
Archaea (% total bacteria)	1.3	1.4	1.8[a]	1.4[ab]	0.9[b]	1.5	1.2	0.1	0.60	0.03	0.40	0.77	0.35	0.17	0.93
Fibrobacter (log10 copies/mL)	6.4	6.6	6.4[a]	6.5[a]	6.7[b]	6.5	6.5	0.0	0.04	< 0.01	0.93	0.10	0.63	0.54	0.09
R. flavefaciens (log10 copies/mL)	5.3	5.4	5.4	5.3	5.3	5.4	5.3	0.0	0.44	0.67	0.18	0.59	0.96	0.12	0.59

Abbreviations: VFA, volatile fatty acids; GP, grape pomace; VP, wild grape pomace; PS, persimmon skin, FRE, fresh material; SIL, silage; -PEG, without polyethylene glycol; +PEG, with polyethylene glycol. [abc] Values within the row with different superscripts are significantly different ($p<0.05$). [1)] Numbers in parenthesis indicate the number of cultivation bottles in the experiment. [2)] The result of post estimation showed that the following pairs were significantly different ($P<0.01$): (CONT,-PEG) vs. (FRE,-PEG), (CONT,-PEG) vs. (SIL,-PEG), (CONT,-PEG) vs. (SIL, +PEG), (CONT, +PEG) vs. (FRE,-PEG), and (FRE,-PEG) vs. (FRE, +PEG).

However, it cannot be responsible for the mitigation of methane production as this effect seemed to depend largely on the carbohydrate profile of the FBs. Owing to the differences between hydrolyzed tannin and CT in terms of effects on fermentation in the rumen, determining the protein binding capacity of tannins present in FBs might be important for assessing the extent to which they would affect rumen microbes (Jayanegara et al., 2009). The finding that no significant effects of tannins on methane generation were observed may be mostly attributed to the limited amount of FB inclusion (one-third to total DM), which was aimed at practical implementation.

Conclusions

The results of the in vitro cultivation experiments indicated that not only the composition but also the ensiling of FBs affected rumen fermentation patterns and the degree of methane generation in in vitro culture. The results suggested that ensiled FBs could initially have some direct or indirect effects on the reduction of methanogens. This is primarily because of the compositional changes in the fibrous fraction during ensiling as well as the presence of readily fermented substrates, whereas tannins in these FBs seemed to have little effect on the in vitro ruminal methane generation. Animal feeding experiments are warranted to determine whether feeding FBs increases feed efficiency owing to the improvement of fiber digestibility, or whether the effect might be offset by increasing methane emission. Detailed monitoring of the digestion kinetics of nutrients, as well as of the microbial interactions within the ecosystem by performing animal experiments, might be needed for the practical application of ensiled FBs as feed for optimized rumen fermentation.

Chapter III

Fermentative quality and animal acceptability of ensiled persimmon skin with absorbents for practical use in ruminant feed

Abstract

Persimmon skin (PS), while representing an attractive feed source, requires an appropriate preservation procedure to increase its shelf life. The fermentation quality, rumen fermentation, and intake of PSS ensiled with different dry absorbents were assessed. Silage on a table scale was prepared (Experiment 1), and five different mixtures were evaluated: PS without an additive, PS plus *L. buchneri* inoculum (LB), and PS plus LB plus each of the absorbents kraft pulp, wheat bran, or beet pulp. The laboratory bags kept at 25 °C, opened at 0, 14, 28, and 60 days for fermentation quality and chemical analysis (n = 3 for each measurement). Further, with an in vitro rumen simulated cultivation study (Experiment 2), the fermentation pattern of PS with a mixture of two absorbents (kraft pulp and wheat bran) was evaluated either raw (no fermentation) or ensiled (n = 4 for each treatment). Finally, an in vivo experiment has been conducted using six dry ewes assigned based on their body weight to two experimental groups in a crossover design of two periods (Experiment 3). The control group fed a 100% basal diet (tall fescue hay and concentrate mixture) and ensiled PS (PSS) group, a 20% DM substitution of tall fescue with PSS mixed with kraft pulp as the sole absorbent. As results of Experiment 1, regardless of the absorbents used, the effluent volume of the lab bags was lower in absorbent-treated groups ($p < 0.001$). In Experiment 2, the condition of the PS with absorbents (raw or ensiled) did not affect the total gas production ($p > 0.05$), but an increased propionate proportion was observed in PSS with absorbents compared to basal diet ($p = 0.019$). The proportion of methane to the total gas in the PSS group was considerably reduced compared with that in the other groups ($p < 0.001$), concurrently showing a

lower proportion of archaea to total bacterial count. In the animal trial (Experiment 3), DM intake was similar between groups ($p > 0.05$), but ewes spent a shorter time eating in the PSS-fed group ($p = 0.011$). The inclusion of the PSS resulted in a significant increase in the digestibility of DM and NDF. No significant difference in the rumen fermentation parameters except for the high proportion for butyrate in the PSS group compared to control. This study suggests the practical use of PSS fortified with dry absorbents as a part of ruminant feed.

Introduction

Persimmon skin silage showed a prominent lactic acid fermentation profile in the previous experiment (Chapter II) when inoculated with *L. buchneri* and ensiled for 28 days. However, the high moisture content remains an obvious obstacle militating against PSS preparation, which resulted in high effluent leakage. In this sense, a variety of feedstuff and non-feed materials including beet pulp (BP), wheat bran (WB), wheat straw, newspaper, and paper waste have been investigated as dry absorbents (Fransen and Strubi, 1998; Okine et al., 2007; Razak et al., 2012). The hypothesis behind this experiment is that the inclusion of dry absorbents can be an effective method to reduce the effluent problem in PSS. In particular, kraft pulp (KP) made from wood chips through a cooking process that selectively removes the lignin has been evaluated as a replaceable feed material for other, more readily, digestible carbohydrate sources (Maeda et al., 2019; Nishimura et al., 2019). Noteworthy was the use of KP as an absorbent for PS silage, which would help optimize carbohydrate balance and therefore has been evaluated as a particularly suitable for initiative solid feed for calves (Kido et al., 2019). In the previous in vitro experiment of PSS without dry absorbents, the reduced methane generation was likely dependent on the carbohydrate profile and the compositional changes in the ensiled PS. Hence it is interesting to evaluate the effect of compositional changes in PSS mixed with absorbent on the in vitro rumen fermentation profile and methane generation.

Furthermore, in vitro assessment does not consider some issues, such as long term microbial adaptation, continuous culture flow, and palatability of the tested material. In particular, PS contains a considerable proportion of certain bioactive compounds such as condensed tannin which produces an astringent sensation in the mouth and may impair the digestibility of nutrients (JETRO, 2016; Krueger et al., 2010). For its practical use as a feedstuff, it is necessary to assess

that no adverse effects on palatability, intake, and digestibility occur. So the purpose of the present study was to evaluate the fermentation quality, rumen fermentation profile (in vitro and in vivo), nutrient digestibility, palatability, and intake of PSS fortified with different dry absorbents

Materials and Methods

Experiment 1. PS Silage

Silage preparation

Persimmon skin was obtained from a local processing provenance in Nagano prefecture, Japan. In this study, three types of commercially available dry absorbents were used: kraft pulp (KP, Nippon Paper Industries Co., Ltd., Tokyo, Japan), wheat bran (WB, Nippon Flour Mills Co., Ltd., Tokyo, Japan), and beet pulp (BP, Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan). *L. buchneri* NBRC107764 inoculum was used (NITE Biological Resource Center, Tokyo, Japan). The experimental treatments were as follows: PS without additive (CONT), PS plus *L. buchneri* inoculum (1.0×10^9 colony forming units (CFU)/kg PS, LB) and LB plus absorbents: 416 g KP/kg PS (DM basis; KP), 788 g WB/kg PS (WB) or 402 g BP/kg PS (BP). The inclusion rate of each absorbent depended on its water retention capacity, based either on previous literature values (WB and BP) (Razak et al., 2012) or on information provided by the manufacturer (KP).

The procedures for bag silage preparation and fermentation control were described in Chapter II. Briefly, PS has been cut to a short length (5 cm–10 cm) before combining the inoculum and absorbents, mixed all materials well to ensure even distribution. Approximately 20 g of each treatment was packed in polyethylene bags (15 cm × 22 cm), which tightly heat-sealed under vacuum (SQ-205S; Asahikasei Packs Co. Ltd., Tokyo, Japan), and then incubated at 25°C. Fifteen bags per group were prepared for this ensiling experiment.

Chemical and microbial analysis of silage

On the day of ensiling (day 0), two bags per treatment were taken for chemical and microbiological analysis. After that, the fermentation profile was evaluated by opening three bags for each group on day 14, day 28, and day 60 of the ensiling process. To detect the fermentation end products, water extract was prepared from silage by adding 180 ml distilled water into bags and homogenizing for 1 min, thereafter storing it for 2 h at 5°C. A portion of the water extract was used to measure the pH using a pH meter (Model D-51, Horiba Co. Ltd., Kyoto, Japan). Another portion from the silage extract was divided into two subsamples. One subsample was centrifuged at 10,000 × g for 10 min, and the collected supernatant was used for the analysis of organic acids by high performance liquid chromatography according to the same protocol described in experiment 2. Batch Culture Trial, and for ammonia nitrogen determination, using a commercial kit (F-Kit Ammonia, Roche Diagnostics, Tokyo). The second subsample (uncentrifuged) was used for microbial counts after serial dilution using a phosphate buffer solution, using the diluted sample to determine LAB counts (using MRS agar [Oxoid, Basingstoke, UK] incubated at 30°C for 3 days), and yeast counts (using chloramphenicol-added potato dextrose agar, incubated at 25°C for 7 days).

Another three silage bags were weighed and opened on day 60. After opening the bags, the gas loss was calculated, as described in the Calculations and Statistical Analysis section, and then the effluent output was measured using a graduated cylinder. Remained contents in the bag were used for the determination of DM and NDF. Dry matter content was determined by oven drying at 100°C for 3 h, also was the condition for DM determination of silage effluent. Methods for proximate analyses (crude ash [942.05], crude fat [945.16], and crude protein [CP, 976.05]) were followed to standards described by AOAC (AOAC, 1990). The NDF was analyzed following the

method described previously (Van Soest et al., 1991) using heat-stable alpha-amylase without the use of sodium sulfite, and presented NDF result, including the ash. Chemical analyses (DM, NDF, and fermentation end-products) were conducted twice for each bag (and its water extract), and the mean value of these two measurements per bag was used for the statistical treatment. For reference information, soluble protein and fiber compartments of test material mixtures were calculated prior to silage fermentation with reference to either proximate analysis data (PS and KP) or a standard table of feed composition (BP and WB) (NARO, 2009) (Table 3.1)

Table 3.1. Detail feed composition values of test feeds prior to ensiling (Experiment 1).

Nutrient	CONT and LB	KP	WB	BP
Soluble protein (g/kg DM)	25	22	34	24
NDF (g/kg DM)	236	331	284	269
ADF (g/kg DM)	95	206	107	117
ADL (g/kg DM)	15	20	19	23
Hemicellulose (g/kg DM)	141	126	177	152
Cellulose (g/kg DM)	80	185	89	93

Abbreviations: CONT, PS without additive; LB, PS plus *L. buchneri* inoculum; KP, LB plus either of 125 g kraft pulp/kg PS; WB, 50 g wheat bran/kg PS; BP, 125 g beet pulp /kg PS. ADF, Acid detergent fiber; ADL, Acid detergent lignin; NDF, Neutral detergent fiber. Hemicellulose = NDF - ADF; Cellulose = ADF-ADL. As mentioned in Materials and Methods, values except for NDF were the calculated ones with reference to those in each material (PS, KP, WB, and BP). The amount of inoculated LAB was excluded from the calculation.

Experiment 2. Batch Culture Trial

Judging from the results of the ensiling experiment, a mixture of KP and WB was applied as the absorbent mixture (ratio approximately 1:1; reason of the mixture was simply due to unavailability of KP in a sufficient amount) for laboratory-scale PSS preparation. 9.2 kg DM of PS (30 kg as fresh matter [FM] weight) was mixed with a 400 g absorbent mixture /kg PS [DM basis]. Before ensiling, a sample of 100 g (FM basis) was taken and air-dried for further in vitro incubation tests. Then, the mixture was ensiled, for this it was inoculated with 1.0×10^9 CFU/kg PS *L. buchneri*, transferred it to a plastic bag (size 45 L), followed by foot treading, and then tightly sealed it. The mix was ensiled in a temperature-controlled room set at 25°C for 60 days (November to January; fermentation properties are available in Table 3.2). Persimmon skin silage sample air-dried at 60°C for 16 hours, ground and kept them at −20°C until using them in the in vitro culture test. The remainder of the lab scale silage was subject to feed animals in the preliminary feeding test described in the next section.

An in vitro incubation experiment was conducted using unfermented and ensiled PS plus absorbents basically followed to those described in Chapter II. Experimental substrates were set as 1) the basal feed group, based mainly on forage material (commercial timothy hay; DM, 850 g/kg FM; CP, 110 g/kg DM; digestible energy, 12.5 MJ/kg DM; calculated value) and concentrate (commercial concentrate; DM, 870 g/kg FM; CP, 220 g/kg DM; DE, 15.5 MJ/kg DM; calculated value) at ratio 80:20 (DM basis); 2) the PS and absorbent group (PSA) containing basal feed with air-dried, unfermented PS with the absorbent (at ratio 82:18 [DM basis] and 67:33 [fresh basis]; adjusted to level in Chapter II; and III) the ensiled PS group (PSS), including basal feed with ensiled PS with the absorbent (at same ratio as PSA). The compositions of PSA and PSS were as follows:

Table 3.2. Chemical composition and microbial characteristics of persimmon skin silage supplemented with absorbents manufactured in a laboratory scale.

Item	For batch culture test (Experiment 2) and Pre-test (Experiment 3)	For Main-test (Experiment 3)
Ensiling duration (days)	60	21
Values post ensiling		
DM (%)	34.0	31.5
pH	3.60	3.60
Yeast (log10 CFU/g FM)	Not detected	Not detected
Lactic acid bacteria (log10 CFU/g FM)	8.33	8.42
Effluent (mL/100g FM)	0	0

Abbreviations: CFU, colony forming unit; DM, dry matter; FM, fresh matter.

DM, 383 g/kg FM; CP, 36 g/kg DM; NDF, 350 g/kg DM for PSA, and DM, 370 g/kg FM; CP, 39 g/kg DM; NDF, 440 g/kg DM for PSS, respectively.

Rumen fluid samples for the cultivation test were collected from three beef steers (Japanese Black, 15 months old, BW 380 kg) fed concentrate (5.0 kg DM/day) and a 1.4 kg DM mixture of forages comprising Italian ryegrass (*Lolium multiflorum*) and orchard grass (*Dactylis glomerata*) via a rumen fistula immediately before the morning feeding. The collected fluids were equally mixed and filtered through four layers of cheesecloth, diluted with artificial saliva (McDougall buffer) at a 2:1 (buffer: the rumen fluid) ratio, and used it within 2 h of collection. A 40 mL aliquot of the diluted rumen fluid was transferred into a 100 mL serum bottle containing experimental substrate (1.0 g), flushed continuously with CO_2 gas. The bottles were sealed with a rubber plug and aluminum cap after flushing the headspace with CO_2 gas and then incubated them at 39°C for

24 h, with periodical removal of headspace gas by a needle attached cylinder. This test was performed in a single run using four replicate bottles.

After incubation, the total headspace gas production, methane proportion, VFA content, and ammonia nitrogen were measured. Headspace gas production was determined by cumulating periodical gas removal. The proportion of methane was analyzed by a gas chromatography system (Shimadzu GC-8A) following to previous report (Abrar et al., 2016), using a steel column (2 m, 3 mm, Shincarbon-ST; Shinwa Chemical Industries Ltd, Kyoto, Japan) and a thermal conductivity detector (TCD, 210°C). Argon gas was used as the carrier gas at a 50 mL /min flow rate. A 0.5 mL aliquot of headspace gas was injected by a gas-tight syringe. Methane gas volume in milliliters was calculated using a standard gas mixture, calculated using an attached software (ChromatoPak). Volatile fatty acids content was analyzed by high performance liquid chromatography, using an LC- 2000 system (JASCO Corporation) under the conditions following to those in the previous report of Kido et al. (2019), under the following conditions: column, Inertsil ODS-3 250 mm × 4.6 mm (GL Science Co. Ltd., Tokyo, Japan); oven, 40°C, mobile phase, 10% acetonitrile 0.02% perchloric acid; flow rate, 1 ml/min; detection, 210 nm absorbance. Operation control, peak detection, and quantification were performed using software attached to the system (ChromNAVI). Ammonia nitrogen was determined by a commercial kit (F-kit, Roche Diagnostics, Basel, Switzerland) according to the instruction.

The total bacterial count, the archaea, and the *Fibrobacter* from the batch culture test were quantified using a real-time polymerase chain reaction method. DNA extraction for microbial analysis was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations, and stored the obtained DNA at −20°C until analysis. For the real-time PCR, the primer sets Eub338F (ACTCCTACGGGAGGCAG) and

Eub522R (ACGTCRTCCMCNCCTTCCTC) for total bacteria count, qmcrA-F (TTCGGTGGATCDCARAGRGC) and qmcrA-R (GBARGTCGWAWCCGTAGAATCC) were used for prokaryotic archaea count, and Fs1f (GTTCGGAATTACTGGGCGTAAA) and Fs1r (CGCCTGCCCCTGAACTATC) for *Fibrobacter* count using CFX96™ Real-Time system (Bio-Rad Inc., Hercules, CA) and a SYBR(R) Premix Ex Taq™ Kit (Takara Bio Inc., Otsu, Japan) for the real-time PCR. Cycling conditions were performed with 40 cycles; each cycle included denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s, followed by dissociation curve analysis to confirm that the expected PCR end products have been obtained

Experiment 3. Feeding Trial

Pretest

Two feeding experiments were conducted to assess its acceptability in partial substitution with PSS. Because there was no comparable data on the provision of PSS (or even PS) on ruminants, a preliminary experiment was firstly conducted (referred to as pretest) on dry ewes. All animal management and handling were conducted following the guidelines of Shinshu University, with the approval for the animal experiment that was the same as above. In this test, six multiparous/primiparous ewes were assigned (Suffolk breed) with an average body weight (BW) of 69 ± 8 kg (mean ± SD) in a 3 × 3 design of two animals each and fed each group one of the following diets (Table 3.3; 11 days for the adaptation and 3 days for the collection of data regarding feed intake): (1) control diet without PSS; or a diet with (2) 12% or (3) 25% DM replacement by the PSS product that was used in the in vitro rumen cultivation test described above.

Table 3.3. Feed compositions for the feeding trial (Pre-test, Experiment 3).

Item	Treatment		
	0 % PSS	12%PSS	25% PSS
Hay cube (g/ kg DM)	586	621	656
Wheat bran (g/ kg DM)	413	191	0
Persimmon skin silage (g/ kg DM)	0	122	245
Soybean meal (g/ kg DM)	0	64	97
DM (g/kg FM)	881	756	661

Abbreviations: PSS, persimmon skin silage. DM, dry matter; FM, fresh matter.

Main test

Based on the observation from the pretest, we decided to mix PSS with the absorbent as 20% DM replacement to evaluate the palatability of the mix and animal behavior toward PSS in the main test. Persimmon skin was collected in November 2018 from the production area in the Nagano Prefecture, Japan. Kraft pulp has been used as dry absorbent and silage was inoculated with *L. buchneri* (1.0×10^9 CFU/kg the ensiled mixture). The freshly collected PS (30 kg as fresh matter [FM] weight) was mixed with 400 g KP/kg DM PS, and packed the mixed material in large doubled polyethylene bags (size 45 L), followed by foot treading, and then tightly sealed it to be used in the feeding trial. The fermentation profile (silage pH, LAB, and yeast count) was monitored once per week as described in the previous section, and values at the endpoint (three weeks) were summarized in Table 3.2.

Six dry ewes were assigned (Suffolk breed) based on BW, with initial BW of 79 ± 7 kg (mean ± SD) to two groups in a crossover design. Each animal was kept individually in a cage

fitted with isolated feed buckets and a water container and supplied every cage with a mineral block (per 100g: 39.9 g Na, 1.33 mg Fe, 106 mg Ca, 102 mg Mg, 0.04 mg Mn, and 56.4 mg K). Each group was fed a ration with either 0% or 20% PSS in two consecutive experimental periods with three animals per treatment (amounting to six animals per treatment). To supply the maintenance requirements for sheep according to the Japanese guidelines, the two diets were formulated to have practically the same level of energy and CP (Table 3.4), by adjusting proportions of the three feed materials (PSS, tall fescue hay, and concentrate mainly consisted of maize, soybean meal, and barley) (AFFRCS, 1996). Before offering it to the animals, tall fescue hay was chopped with a mechanical chopper to 8 cm. The diets were distributed twice per day at 0800 h and 1600 h, collecting and weighing feed refusals every day. Each period extended for 14 days and included 10 days for adaptation, followed by 4 days for measurement and collection. During the adaptation period, the amount of PSS was gradually increased to 6% (1st and 2nd day) and 13% (3rd and 4th day) to substitute tall fescue hay, up to 20% on a DM basis (after 5th day). Interval for washout was set for three days between diet switching. Rumen liquor samples were collected from animals via a flexible stomach tube with a vacuum pump (ULVAC Kiko, Inc. DAP-15) before morning feeding on the last day of each period. The tube was washed using freshwater between sample collections. Macroscopic analysis of the ruminal fluid was conducted immediately after collection, including ruminal fluid pH, odor, color, and viscosity. Ruminal liquor was strained through four layers of gauze, and subsamples were frozen at -20 °C until further analyses. Total bacterial count and archaea were quantified with qPCR using the same method described in batch culture test, similarly for the VFAs and ammonia nitrogen. Fecal samples were collected when ewes naturally defecated, or samples were obtained from the rectum of each ewe twice per day, before morning feeding and in the afternoon.

At the end of the collection period, fecal samples thawed and homogenized, forming composite samples per animal. For determination of the apparent digestibility of DM and NDF, fecal aliquot samples from each animal were pre-dried in a forced-air oven at 65°C for 72 h and ground in a Wiley mill to 2-mm particles for analysis of acid-insoluble ash (AIA). Acid insoluble ash in feces and feed was determined according to Van Keulen and Young (1977), with slight modification. Briefly, 5 g of previously dried feces or feed were weighed in porcelain crucibles and ashed at 600°C for 6 h in a muffle furnace (Model FM38, Yamato Scientific Co., Ltd.). These were then boiled in 2 N hydrochloric acid (HCl) on a hotplate for 10 min before being filtered through ashless filter paper (Whatman No 41) and then ashed again at 600°C for 6 hours. The samples were weighed after the second ashing to detect the AIA percentage. Dry matter and nutrient digestibility were calculated as following:

DM digestibility (%) = (1-A/B) × 100

Nutrient digestibility (%) =[1-(A/B) x (N_B /N_A)] × 100;
where A and B were the AIA concentrations in the feed and feces, respectively.
N_A and N_B were the nutrient concentrations in the feed and feces, respectively.

For behavior measurement, time spent eating was recorded by direct (i.e., without camera usage) monitoring for two hours after each feed offering timing. For other timing periods, two sets were applied of portable time-lapse cameras with Illumi-Night Sensors to facilitate video capturing during the nighttime; each one recorded the behavior of three animals. Cameras were set on motion detector mode for three days to capture their behavior in each experimental period, to define time spent eating, and the onset of a rumination bout as the time when regurgitation occurred namely, when a bolus came up the esophagus and reached the mouth. The end of a rumination bout was the minute the last bolus was swallowed.

Calculations and Statistical Analysis

In Experiment 1, gas loss of silage bags after 60 d of ensiling was calculated as follows:

$$\mathrm{GL\ (g)} = \mathrm{BWE\ (g)} - \mathrm{BWO\ (g)} \quad (1)$$

Where GL is the gas loss (g), BWE is the bag weight at the ensiling (g), and BWO is the bag weight after the opening (g).

Analysis of variance (ANOVA) was applied for pH, yeast, lactic acid bacteria, DM, and NDF in the ensiling experiment. The following model was used:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ij} \quad (2)$$

Where Y_{ijk} = observations for dependent variables; μ = overall mean; α_i = the fixed effect of treatment; β_j = the fixed effect of time; $(\alpha\beta)_{ij}$ = the interaction between treatment and time; and e_{ij} = the residual error. Numbers of j were different among items (four for pH, yeast, and lactic acid bacteria; two for DM and NDF). The data of the in vitro cultivation and of the fermentation characteristics and chemical composition at the endpoint (60 days) except for those described above were analyzed using a one-way ANOVA and Tukey's test to detect the presence of significant differences between treatment means. All results from the in vivo trial (main test of Experiment 3) were stated as the mean ± SE. Wilcoxon signed-rank tests were applied for comparisons between order effects and period effects but found no significant difference. Differences between means were analyzed with independent samples t-test. All statistical procedures were conducted using Statistical Packages for the Social Sciences (SPSS). A p-value of less than 0.05 was considered statistically significant.

Table 3.4. The chemical composition of the main dietary ingredients and tested feed in the feeding trial (main test, Experiment 3).

Nutrient	PSS	Tall Fescue Hay	Concentrate Mixture	Test Feed	
				Control Period	PSS Period
DM (g/kg FM)	320	890	870	884	668
OM (g/kg DM)	945	935	960	941	943
CP (g/kg DM)	30	45	220	90	85
CFat (g/kg DM)	16	10	25	14	15
NDF (g/kg DM)	533	650	100	540	517
NFC (g/kg DM)	366	230	615	297	326
TDN (g/kg DM)	580	500	840	580	590
DE (MJ/kg DM)	10.9	9.2	15.6	10.8	11.0

Abbreviations: CFat, crude fat; CP, crude protein; DE, digestible energy; DM, dry matter; NDF, neutral detergent fiber; NFC, non-fiber carbohydrates (calculated by OM - [sum of CP, CFat, and NDF]); OM, organic matter; PSS, persimmon skin silage; and TDN, total digestible nutrients. DE and TDN were calculated in accordance with a feeding standard (NARO, 2009). For the concentrate mixture, a commercial concentrate for dairy heifers was used consisted mainly of corn, barley, soybean meal, rapeseed oil meal, wheat bran, calcium bicarbonate, and vitamins. Test food was an example ration for 80 kg BW ewes; compositional values were calculation results based on those in each material

Results

Experiment 1. PS Silage

As shown in Table 3.5, BP-treated silage had a lower pH than that in other groups at the beginning of the ensiling process. After 14 days of ensiling, the pH dropped dramatically to less than 4.0 for all treatment groups. After 60 days of ensiling, a higher pH in the WB-treated group was detected compared to that of the other groups ($p < 0.001$). While a certain volume of lactobacilli in control was determined, *L. buchneri* inoculation resulted in an increased LAB count relative to the control group ($p < 0.001$). Moreover, in WB and BP, the yeast count was under the detection level ($\times 10^2$ cfu/g) from 14 days. After 60 days of ensiling, no yeast growth was detected in any LB-treated groups, with or without absorbents.

Only lactate and acetate were detected, but neither propionate nor butyrate was noted in any of the silages (Table 3.6). Lactate levels were significantly higher in WB and BP than in other groups. Acetate levels in the LB-inoculated groups (with or without the absorbents) were marginally higher than those in the control group. No significant difference was detected in terms of ammonia nitrogen concentration between the groups.

Dry matter and NDF of each group before and at 60 days of silage fermentation are shown in Table 3.6. Regarding other nutrients, the initial CP content (g/100 g DM) was 3.6±0.1, 3.7±0.2, 3.2±0.2, 6.4±0.3, and 3.4±0.2 for CONT, LB, KP, WB, and BP, respectively, but after fermentation CP content was not traced. Initially, the inclusion of KP, WB, and BP increased the DM content compared with the control and LB groups. The NDF proportion seemed to increase in the three absorbent groups (KP, WB, and BP) at the beginning ($p < 0.001$). The NDF proportion per DM decreased in the CONT, LB, and BP groups compared with that pre-ensiling, whereas it increased

during ensiling in the KP group. The absorbent-treated silages (KP, WB, and BP) produced a negligible amount of effluent, whereas the other groups (CONT and LB) generated up to 21.3 mL (CONT) and 17.5 mL (LB) of effluent per 100 g FM, respectively, accounting for 12 % DM loss in the effluent. The control group, compared with two of the absorbent-treated groups (KP and BP), had the highest gas production during ensiling ($p = 0.045$).

Experiment 2. Batch Culture Trial

No significant difference was determined in the total gas production ($p = 0.118$) and in the total VFA production ($p = 0.108$) among groups (Table 3.7). Propionate proportion in total VFA significantly increased in PSA and PSS groups compared to that in the control ($p = 0.019$), a significant decrease in acetate proportion was observed in PSA group ($p < 0.001$) compared to control and PSS. In the PSA group, a significant decrease was noted in ammonia, in comparison with that of the control concentration ($p = 0.004$). In PSS, the proportion of methane to total gas was considerably reduced in relation to that in the other groups ($p < 0.001$). Compared with the fresh material, PSS showed a lower proportion of archaeal 16S rRNA in the total community ($p = 0.006$), and also a lower proportion of *Fibrobacter succinogenes* ($p < 0.001$).

Table 3.5. Time course of fermentation characteristics (pH, yeast, and lactic acid bacteria) of persimmon skin supplemented with absorbents during ensiling.

Ensiling period	Treatment					Contrast
	CONT	LB	KP	WB	BP	
			PH			
Day 0	6.32±0.07 ^{aA}	6.27±0.02 ^{aA}	6.37±0.04 ^{aA}	6.31±0.01 ^{aA}	5.79±0.03 ^{bA}	Trt, P < 0.001 Time, P < 0.001 Trt×Time, P < 0.001
Day 14	3.72±0.02 ^{aB}	3.63±0.02 ^{aB}	3.68±0.02 ^{aB}	3.50±0.03 ^{bC}	3.71±0.01 ^{aBC}	
Day 28	3.63±0.04 ^{bcB}	3.57±0.01 ^{cBC}	3.68±0.03 ^{bB}	3.58±0.01 ^{cC}	3.76±0.02 ^{aB}	
Day 60	3.58±0.04 ^{cB}	3.53±0.02 ^{cC}	3.56±0.01 ^{cC}	3.78±0.01 ^{aB}	3.68±0.01 ^{bC}	
			Yeast (log10 CFU/g FM)			
Day 0	4.53±0.05 ^{B}	4.38±0.01 ^{A}	4.25±0.05 ^{A}	4.43±0.02 ^{A}	4.23±0.07 ^{A}	Trt, P < 0.001 Time, P < 0.001 Trt×Time, P < 0.001
Day 14	5.35±0.40 ^{aA}	3.18±0.18 ^{bB}	2.05±0.05 ^{cC}	ND ^{dB}	ND ^{dB}	
Day 28	5.00±0.70^{aAB}	3.38±0.02 ^{bB}	2.51±0.03 ^{bB}	ND ^{cB}	ND ^{cB}	
Day 60	4.25±0.23 ^{aBC}	ND ^{bC}	ND ^{bD}	ND ^{bB}	ND ^{bB}	
			Lactic acid bacteria (log10 CFU/g FM)			
Day 0	6.14±0.12 ^{cC}	7.27±0.03 ^{aC}	7.35±0.04 ^{aC}	6.93±0.00 ^{bC}	7.32±0.03 ^{aC}	Trt, P < 0.001 Time, P < 0.001 Trt×Time, P < 0.001
Day 14	7.89±0.02 ^{cA}	8.52±0.09 ^{aAB}	8.95±0.03 ^{aA}	8.37±0.04 ^{bA}	8.09±0.07 ^{bAB}	
Day 28	7.77±0.07 ^{cA}	8.86±0.02 ^{aA}	8.92±0.05 ^{aA}	8.04±0.07 ^{bB}	8.17±0.06 ^{bA}	
Day 60	6.48±0.17 ^{cB}	8.45±0.01 ^{aB}	8.50±0.04 ^{aB}	8.15±0.05 ^{bB}	7.85±0.09 ^{bB}	

Abbreviations: CONT, PS without additive; LB, PS plus L. buchneri inoculum; KP, LB plus either of 125 g kraft pulp/kg PS; WB, 50 g wheat bran/kg PS; BP, 125 g beet pulp /kg PS. CFU, colony forming unit; FM, fresh matter. Values are expressed as mean ± SE; mean values in the same row with different small superscripts are significantly different, assuming ND as 0; mean values in the same column with different large superscripts for each item are significantly different, assuming ND as 0.

Table 3.6. Fermentation characteristics of persimmon skin silage supplemented with absorbents (Experiment 1)

Items	Treatment					Contrast
	CONT	LB	KP	WB	BP	
DM (g/kg FM)						
Day 0	288 ± 5 ^{c}	280 ± 5 ^{c}	365 ± 6 ^{b}	409 ± 3 ^{a}	359 ± 7 ^{b}	Trt, $p < 0.001$ Time, $p = 0.07$
Day 60	281 ± 14 ^{c}	261 ± 9 ^{c}	323 ± 26 ^{b}	388 ± 31 ^{a}	355 ± 4 ^{b}	Trt×Time, $p = 0.72$
NDF (g/kg DM)						
Day 0	236 ± 4 ^{c}	236 ± 4 ^{c}	377 ± 11 ^{a}	336 ± 3 ^{b}	327 ± 6 ^{b}	Trt, $p < 0.001$ Time, $p = 0.73$
Day 60	196 ± 62 ^{cd}	167 ± 34 ^{d}	494 ± 23 ^{a}	338 ± 21 ^{b}	287 ± 37 ^{c}	Trt×Time, $p = 0.06$
After 60 days ensiling						
Lactate (g/100 g DM)	3.51 ± 0.19 ^{b}	3.39 ± 0.17 ^{b}	2.99 ± 0.11 ^{c}	4.39 ± 0.29 ^{a}	4.88 ± 0.31 ^{a}	$p = < 0.001$
Acetate (g/100 g DM)	3.52 ± 0.09	6.07 ± 0.27	4.15 ± 0.07	4.76 ± 1.03	5.16 ± 1.47	$p = 0.08$
NH_3-N (mg/g Total N)	0.31 ± 0.11	0.20 ± 0.18	0.09 ± 0.07	0.06 ± 0.07	0.48 ± 0.15	$p = 0.07$
Effluent (mL/100 g FM)	21.3 ± 1.3 ^{a}	17.5 ± 2.5 ^{a}	0 ^{b}	0 ^{b}	0 ^{b}	$p < 0.001$
DM loss in effluent (g/100 g DM)	12.1 ± 0.4 ^{a}	12.0 ± 0.5 ^{a}	0 ^{b}	0 ^{b}	0 ^{b}	$p < 0.001$
Gas (g/100 g FM)	0.37 ± 0.02^{a}	0.33 ± 0.03 ^{ab}	0.22 ± 0.03^{bc}	0.27 ± 0.04^{abc}	0.17 ± 0.04^{c}	$p = 0.045$

Abbreviations: CONT, PS without additive; LB, PS plus L. buchneri inoculum; KP, LB plus either of 125 g kraft pulp/kg PS; WB, 50 g wheat bran/kg PS; BP, 125 g beet pulp /kg PS. DM, dry matter; FM, fresh matter; N, nitrogen; NDF, neutral detergent fiber; and Trt, (effect of) treatment. Values are expressed as mean ± SE (n = 2 for day 0, and n = 3 for others); mean values in the same row with no common superscripts are significantly different.

Table 3.7. Effects of including ensiled or fresh persimmon skin (PS) on the in vitro incubation characteristics (Experiment 2).

Items	BD	PSA	PSS	P
Total gas production (mL)	13.9 ± 1.5	16.6 ± 0.9	15.1 ± 1.3	0.118
Total VFA (mmol/L)	91.6 ± 7.5	80.5 ± 4.8	94.2 ± 8.2	0.108
Acetate (mol %)	52.3 ± 1.2 ^{a}	46.7 ± 0.2 ^{b}	52.4 ± 0.4 ^{a}	< 0.001
Propionate (mol %)	35.3 ± 2.6 ^{b}	39.9 ± 1.3 ^{a}	40.6 ± 0.8 ^{a}	0.019
Butyrate (mol %)	12.2 ± 3.5 ^{a}	13.3 ± 1.2 ^{a}	6.9 ± 0.5 ^{b}	0.019
NH_3-N (mg/L)	35.8 ± 1.4 ^{a}	18.6 ± 5.5 ^{b}	32.3 ± 3.3 ^{ab}	0.004
Methane (mmol/L)	10.3 ± 1.1 ^{a}	10.1 ± 0.7 ^{a}	1.7 ± 0.4 ^{b}	< 0.001
Total bacteria(10^9 copies/mL)	4.92 ± 0.98 ^{a}	2.67 ± 0.25 ^{b}	4.64 ± 1.16 ^{a}	0.037
Archaea (% total bacteria)	3.33 ± 1.02 ^{ab}	6.64 ± 1.62 ^{a}	1.79 ± 0.60 ^{b}	0.006
Fibrobacter (% total bacteria)	1.93 ± 0.40 ^{b}	2.88 ± 0.38 ^{a}	0.93 ± 0.15 ^{c}	< 0.001

Abbreviations: BD, basal diet; PSA, basal diet with unfermented PS (with absorbents) at ratio 67:33; PSS, basal diet with ensiled PS (with absorbents) at ratio 67:33; and VFA, volatile fatty acid. Values are expressed as mean ± SE; mean values in the same row with different superscripts are significantly different.

Table 3.8. Feed intake, nutrient digestibility, and ingestive behavior in animal experiment using persimmon skin silage (PSS) (main test, Experiment 3).

Items	Control period	PSS period	*p*
DM intake (kg/day)	1.29 ± 0.10	1.40 ± 0.04	0.441
Organic matter intake (kg/day)	1.21 ± 0.09	1.31 ± 0.04	0.428
Crude protein intake (g/day)	95.9 ± 7.0	100.9 ± 3.1	0.693
NDF intake (g/day)	723.6 ± 62.9	747.3 ± 21.7	0.905
DM digestibilty (%)	65.7 ± 6.5^{b}	75.3 ± 2.8^{a}	0.004
NDF digestibility (%)	61.1 ± 3.9^{b}	76.3 ± 3.3^{a}	0.001
Time spent eating (min/kg ingested DM)	164 ± 5 ^{a}	130 ± 8 ^{b}	0.011
Time spent ruminating (min/kg ingested DM)	414 ± 11	413 ± 14	0.950
Body weight change in each period (kg)	-1.00 ± 1.05	-1.50 ± 0.62	0.717

Abbreviations: DM, dry matter; NDF, neutral detergent fiber. Values are expressed as mean ± SE of six animals; measured data for three days in each treatment of each animal were averaged; mean values in the same row with no common superscripts are significantly different.

Experiment 3. Feeding Trials

Persimmon skin silage employed in Experiment 3 was comparable in quality to those of bags of KP group in Experiment 1, as well as a laboratory scale preparation for the preliminary feeding test (Table 3.2). In addition, no effluent leakage was detected in the silage package. In the pretest, no feed refusal was observed during the entire experiment when PSS was offered (up to 25% DM), and daily DM intake did not differ (p = 0.582, one-way ANOVA) among the groups (1.27 ± 0.06 kg/day, mean ± SE).

During short-term experiment (main test), the dietary treatment had no significant effect on BW change. PSS inclusion in sheep feed did not affect DM, CP, and NDF intake (Table 3.8). The apparent digestibility of DM and NDF was significantly higher ($P<0.05$) in the PSS group compared to the control. The result indicated that the PSS-fed group spent less time eating per DM ingestion ($p = 0.011$), but no significant difference in rumination time per DM ingestion.

The dietary treatment did not change pH and the other macroscopic characteristics of the rumen fluid. No significant ($p>0.05$) difference was observed in total VFA, acetate, and propionate molar proportion among control and PSS. Butyrate proportion increased in the PSS group ($P<0.05$) compared to CONT. Ammonia nitrogen in the rumen liquor was within (0.13-0.14mg/mL) and did not show a significant difference among the two groups ($p>0.05$). No significant difference was detected among the groups in the total bacterial and total Archaeal 16S rRNA gene copies

Table 3. 9 Ruminal fermentation parameters of dry ewes fed a diet with or without PSS (main test, Experiment 3)

Item	Control	PSS	P value
Total VFAs (mmol/ml)	77.0 ± 8.1	64.5 ± 5.7	0.13
Acetate mol%	73.5 ± 1.1	70.2 ± 1.9	0.11
Propionate mol%	16.8 ± 0.7	15.2 ± 0.5	0.07
Butyric acid mol%	9.7 ± 0.7^{b}	14.6 ± 1.5^{a}	0.02
NH_3-N(mg/ml)	0.14 ± 0.0	0.13 ± 0.01	0.3
Total bacterial count (log10 copies/mL)	8.0 ± 0.3	7.9 ± 0.1	0.3
Archaea (log10 copies/ml)	7.7 ± 0.0	7.9 ± 0.2	0.13
Ruminal macroscopic analysis			
PH	7.33 ± 0.00	7.30 ± 0.01	0.1
Odor	Aromatic	Aromatic	
Viscosity	Thick	Thick	

Abbreviations: PSS; persimmon skin silage, VFA; volatile fatty acids. Values are expressed as mean ± SE; mean values in the same row with different superscripts are significantly different.

Discussion

Experiment 1. PS Silage

Preventing effluent output comprises two approaches: increasing DM at pre-ensiling, and absorbing water leaked during ensiling. The former idea has been examined in preceding studies, for instance, Fransen and Strubi (1998) evaluated absorbents such as rolled barley, beet pulp, or crushed alfalfa cubes to non-wilted grass forage silage, and found a negative, quadratic relationship between pre-ensiled DM and grass silage effluent. Another group reported that the addition of 9% of bran to vegetable waste (12% DM) decreased effluent output (Özkul et al., 2011). Maintaining silage at high DM also contributes to preventing toxigenic fungi and mycotoxins (Wambacq et al., 2016).

Taking results obtained in Chapter II into account, one silage inoculant has been applied, *L. buchneri* culture. *Lactobacillus buchneri* is known as having the unique ability of anaerobically converting lactic acid to acetic acid and 1,2-propanediol both of which are known as antifungal agents (Oladosu et al., 2016); thus, it is regarded as an effective silage inoculant that prevents aerobic deterioration (Gandra et al., 2017; Nishino et al., 2004; Tabacco et al., 2011). In the present study, LAB inoculation may have affected silage fermentation pattern in either lactate, acetate, or both, compared with the result in CONT. Further, two other metabolites, 1-propanol, and propionate acid, are sometimes found in *L. buchneri* treated silages (Holzer et al., 2003). According to previous report (Krooneman et al., 2002), these compounds are generated from 1,2-propanediol by *Lactobacillus diolivorans*, which is taxonomically close to *L. buchneri*. While propionate in PSS could not be detected, it is worth analyzing detailed lactobacilli population in species level, in order to conceive what is probable mechanism to prevent silage deterioration. In contrast, gaseous loss was particularly high in the absorbent-free groups, a result partly due to low DM (i.e., high moisture content), which promoted unnecessary fermentation by other bacteria, yeast, and fungi (Razak et al., 2012). As PS

includes a high proportion of readily fermentable sugars (Uyeno et al., 2016b), to implement another option in addition to LAB would be desirable for preventing the silage from microbial deterioration. In this regard, supplying absorbent to PS was an effective mean to increase DM. The addition of dry absorbents, because of water retention capacity, successfully reduced effluent volume to zero and in a prevention of DM loss in the effluent that accounted for around 12 g/100 g DM, which presumably involved soluble sugar and protein, and some insoluble nutrients described below. One thing of some importance was that this material has been cut into around 5-10 cm in the bag test, retaining larger surface area per material weight compared to that of practically generated PS having a length of 50-80 cm. This difference would have enhanced effluent output in CONT group.

Notably, both of treatment (absorbent inclusion) and the ensiling process affected the chemical composition of the ensiled material. Although not significant, a relationship between treatments and analysis timings (pre-ensiling and at day 60) in NDF was remarkable. At day 60, NDF decreased in CONT and LB groups, possibly because of hemicellulose breakdown in PS portion (accounted for 141 g/kg DM [Table 3.1]) caused by hydrolysis resulting from fermentation acid. Part of such hemicellulose-derived small molecules presumably dripped off with leaked water. In the case of BP group an NDF decrease was also remarkable, possibly due to degradation of the hemicellulose compartment in BP. On the other hand, as KP comprises almost 100% cellulose (Maeda et al., 2019) and contributed to increase in cellulose proportion (up to 185 g/kg DM [Table 3.1]), which could escape intensive degradation during ensiling, the relative proportion of NDF in total DM was supposed to increase after fermentation.

The ammonia nitrogen level was also low regardless of inoculant and absorbent inclusion, a result in agreement with that of a previous study (Kim et al., 2013) reporting that barley silage treated with fermented persimmon extract had low ammonia nitrogen concentration. It was likely because of

relatively little proportion of CP in PS (around 30 g/kg DM). On the other hand, it may also be due in part to the action of tannins in PS, which have a high affinity to bind protein and protect it from proteolytic microorganisms (Uyeno, 2015).

Overall, all three kinds of absorbents contributed to an improvement in ensiling quality in PS with respect to lowering both gas production and effluent. Judging from results of the ensiling experiment, three kinds of absorbents tested has been assumed to possess essentially similar properties from a practical point of view. Accordingly, in larger scale silo preparations the same inoculum was used, and KP was included as the main composite of the absorbent to make the silage balanced in view of carbohydrate proportion (fiber and readily fermentable sugar).

Experiment 2. Batch Culture Trial

This experiment aimed to reveal the change in in vitro rumen culture when PS with absorbent was mixed to a forage-based feed ration and to elucidate the effect of the ensiling of the PS-absorbent on the cultivation. Interestingly, some changes in the incubation parameters were observed between groups of diets with ensiled- and raw- PS-absorbents (Table 3.7). The PSA contained raw PS plus absorbents (KP and WB in this case), all of which were not yet degraded by ensiling. In Chapter II, the addition of raw PS into diet affected in vitro rumen incubation product decreasing acetate and increasing propionate and butyrate proportions. These results were basically in accordance with results in PSA group of the present experiment (i.e., PS plus KP and WB), although the proportional increase in butyrate was not observed. A significant decrease in ammonia in the PSA group was better attributed to the effective utilization of nitrogen source by bacteria using energy source (fermentable carbohydrates) remaining in the fresh material, rather than the effect of tannin (Krueger et al., 2010).

A decrease in in vitro methane generation brought by PSS group was maintained, as it was reported previously as well, even when absorbents were added. It was in good accordance with low proportion of archaeal 16S rRNA in the total community. As previously explained in Chapter II, partially-degraded fiber generated from ensiling may provide more suitable fermentation substrates for some bacteria involved in fiber digestion, introducing changes in the fermentation product to limited hydrogen generation, which was expected to reduce methane production. Also, in an in vivo trial (Kido et al., 2019), feeding KP to calf resulted in a significant increase in *Fibrobacter* proportion; a similar result was obtained in PSA group. However, the *Fibrobacter* proportion decreased in PSS, which likely had relatively higher NDF content per DM than the other two groups. As recently shown in vivo and in vitro digestibility evaluation trials of fermented feeds containing food byproducts (Cao et al., 2012, 2010b; Chao et al., 2016), the ensiling process improved fiber digestibility; similar change was expected to occur in PSS compared with PSA (before ensiling matter). Taken together, although the detailed fiber profile (ADF and acid detergent lignin) was not determined, it was supposed that during ensiling, the absorbent material changed proportion in structural and non-structural carbohydrates particularly in the case of KP, thereby affecting the rumen microbial profile. When the *Fibrobacter* population was low after incubation, fiber degradation could mainly be participated by other kinds of fiber-degrading bacteria for instance, *Ruminococcus* species. Because *Ruminococcus* is known to release H_2 as a metabolite of cellulose breakdown (Russell and Rychlik, 2001), the hydrogen may have reduced CO_2 to methane, which is, however, inconsistent with present results of decreasing methane generation in PSS. Another interpretation of this result would be that other types of rumen bacteria were activated and collaborated together to degrade various nutrients, including fiber, as it usually occurs in the actual rumen community (Dehority, 2003b). Therefore, the ensiling of PS plus

absorbent might offer favorable conditions for the growth of non-fiber-degrading bacteria that preceded fiber-degrading bacteria, and it would invoke more rigorous fermentation.

Experiment 3. Feeding Trial

In the pretest, offering the persimmon skin silage as a part of the sheep ration did not affect the acceptability of the diet. Basically, as PS contains a high amount of readily fermentable carbohydrates, care to induce sufficient rumination is required when it is fed to replace forage, as animals that received an insufficient quantity of hay spent less chewing time. From this viewpoint, including fibrous materials such as KP and BP may be favorable to alleviate decrease in fiber contents of PS included feed. In the present study, we aimed to determine the availability of PSS by adding absorbents for ruminant feed without animal refusal and found it probable to feed to a certain amount.

In addition, the palatability evaluation was performed for studying the effect of partial substitution (20%) of DM content by PSS (main test). A thorough search of the relevant literature yielded only one animal feeding study conducted by Kim et al. (2006), who demonstrated that when feeding finishing pigs diets containing up to 7% fermented persimmon shell, which positively affected measurements regarding both growth performance and meat quality. In the present study, however, no remarkable changes were detected in the body weight of animals in either group since adult ewes were applied. Sheep spent a significantly shorter time for eating in the PSS-fed period than the control period. There are some probable reasons of good palatability for animals; a high proportion of readily fermentable carbohydrates (as partly explained rich proportion of NFC shown in Table 3.4) may have remained in PSS, although it was expensed during ensiling at a certain amount. Another reason of the palatability would be simply higher moisture content in PSS ration, which helped smooth intake by animals. These factors may have exceeded negative factors within PS, such as astringency. No

significant change in the rumination time per DM intake was likely due to the same level of NDF intake between the groups, partly attributed to fortifying fiber from the absorbent. From this viewpoint, including fibrous materials such as KP may be a favorable option to alleviate decreases in fiber contents of feed that include a certain amount of PS.

Based on results obtained from batch culture and available information on the impact of other plant materials containing phytochemicals compound on ruminal fermentation (Bryszak et al., 2019), the DM replacement of tall fescue with PSS would result in a change in the rumen fermentation pattern in sheep. However, no significant differences were observed between the groups in ruminal pH, total VFA, and the main VFA proportions (acetate and propionate). The lack of such effects may be attributed to the limited amount of PSS inclusion about 20 % of total DM, also indicated to the negligible effect of tannins on rumen ecology. The estimated amount of tannin intake in the current study was 16.8 g/kg DM. The dietary tannins at a level of 20 g/kg DM or less, had little or no influence on VFA concentration (Malik et al., 2017). Aguerre et al. (2016) found that tannins supplemented at (0%, 0.45%, 0.9%, and 1.8 % dietary DM) did not affect total VFA or proportions of acetate and propionate in dairy cattle. The current finding was in contrast with Jayanegara et al., (2012b), who mentioned that the condensed tannin modifies the VFA molar proportions, mainly the ratio of acetate: propionate. Similarly, the overall ruminal bacterial count and methanogen was not significantly changed. The microbial populations in the rumen and hindgut can be affected by tannins (Jones et al., 1994; Molan et al., 2001; Smith et al., 2003). However, previous studies have shown that ruminal bacteria can tolerate tannins (Brooker et al., 1994; Nelson et al., 1998, 1995) and even hydrolyze them (Goel et al., 2005). This elucidates the higher butyrate proportion in the PSS group, which can be resulted from degradation of tannin to butyrate by enzymes secreted from some microbial consortia through 3-hydroxy-5-oxohexanoate pathways (Bhat et al., 1998; Krumholz and Bryant, 1986). Also,

Krueger et al. (2010) observed an increase in the butyrate proportion when fed tannin at a level of 1.5% to Feedlot cattle. The apparent digestibility of DM and NDF was higher in the PSS group compared to the control one which may be evidence of the ensiling effect by increasing the degradability of the fibrous portion. Detailed investigation of the rumen microbiota by the pyrosequencing analysis will be reasonable.

Conclusions

Sequential experiments conducted in this chapter successfully demonstrated PS for practical use as part of the ruminant feed. Using various dry absorbents for moisture adjustment of PSS decreased the effluent loss to a negligible volume. Ensiled PS with absorbent exhibited potential for decreasing methane generation in an in vitro rumen incubation. Moreover, silage fermentation of PS with absorbent may invoke alteration in rumen bacteria and archaea both involved in fiber degradation. Sheep consumed a ration partially substituted with PSS (up to 20%) without adverse effect on feed intake and palatability, ruminal fermentation parameters, and digestibility. Detailed animal feeding trials are required to determine whether feeding PS affects feed efficiency or how it impacts enteric methane emission.

Chapter IV

General Discussion

Nowadays, the great challenge to increase feed production to meet the steady demand for meat and milk has been accompanied to detecting an effective means to settle GHG from ruminants. The current data suggested FBs as a suitable approach for both ends of the problem and indicated the procedures that secure the use of the FBs as sustainable and reliable animal feed. Stepwise experiments have been conducted, 1) to extend FBs shelf life by choosing the suitable preserving method (e.g., silage) and improving the silage fermentative and nutritional quality by testing the effect of different additives (Chapter II and III), 2) to optimize the dose of inclusion and evaluate the efficiency of ensiled FBs on mitigating methane emission from ruminants using in vitro evaluation (Chapter II), 3) to reveal the ingestive response of animal and the impact on ruminal fermentation parameters of adult ruminants which are fed with diets that include PSM-rich fruits by-products through the in vivo evaluation (Chapter III).

To make a clear discussion, the discussion about silage quality from chapter II and III will be combined. In line with the hypotheses, the ensiling of the FBs successfully ensured the long term preservation method. The use of the bacterial inoculum (*L. buchneri*) before ensiling particularly in the case of the PS was a viable plan. Where favorable variations in the pH, fermentation acid content and microbial numbers (LAB and yeast populations) were noticed in *L.buchneri*-treated silages with or without absorbent. These changes were all essential indicators of the silage fermentation quality (Ashbell et al., 1987; Jonsson and Pahlow, 1984). Results confirmed the role of the heterofermentative *L.buchneri* built on the existing evidence from wide literature about their ability in improving the aerobic stability during the feed out stage (Filya, 2003; Kleinschmit et al., 2005; Muck et al., 2018; Tabacco et al., 2011). The relatively high load of the yeast in VP silage was associated with good

aerobic stability, and that did not fit with the theory that the silage with a high yeast population (>10^5 cfu/g) deteriorated as soon as oxygen becomes available (McDonald et al., 1991). These findings were in agreement with Nishino et al. (2004) who reported that aerobic stability in silage was obtained even with more than 10^6 cfu/g of yeast counted at silo opening. This proposes that the loss of aerobic stability has no significant relationship with yeast counts but may be affected with other causes such as the inoculum used, the dominant yeast species in the silage, or some chemical compounds (e.g., polyphenols) present in fermented material. With the focusing on the PSS, even with the high soluble carbohydrate of unfermented PS which makes it from the theoretical point of view ideal for the ensiling process, the pre-ensiling characteristics, mainly high moisture content is an obvious obstacle. This study indicated that the effluent production of ensiled materials is highly affected by, and negatively correlated to, DM content. Upward adjustments of the DM of pre-silage material significantly decreased gaseous loss and effluent output in agreement with the previous studies (Gandra et al., 2017; Jones and Jones, 1996; Okine, 2007; Özkul et al., 2011). This means that DM losses associated with the effluent output and the gaseous loss will consequently decrease resulting in reserving the nutritive value of the ensiled material and avoiding another expected source of pollution (i.e., effluent). The water-polluting potential or biological oxygen demand (BOD, the potential for the removal of oxygen from water) of the silage effluent exceeds the other agricultural pollutants such as pig slurry, cow urine, and cow slurry (90,000, 35,000, 19,000, and 5,000 mg O_2/L respectively (Okine, 2007). Furthermore, providing non-forage fiber sources (KP, WB, and BP) that are rich in fiber (like forages) and are rapidly passed from the rumen (like concentrates) can improve productivity and health of ruminants and control feed costs. Thus, the application of the inoculum and absorbent is highly recommended to improve the nutritional and fermentative PSS quality.

It was needed to optimize the dose of inclusion of FBs and evaluate its efficiency on methane mitigation by the in vitro means (Chapter II). The batch culture evaluation has been suggested as it represents a cheap and simple method and has been widely used to screen and evaluate various feedstuffs and feed additives (Eun et al., 2007). Although the in vitro technique has some limits to epitomize in vivo trials, it was suitable for the evaluation of different feedstuffs for their antimethanogenic activity. In vitro cultivation of the aforementioned FBs showed that the ensiling of these by-products, as well as its composition, was capable of modifying the rumen fermentation and methane generation. The first cultivation experiment in Chapter II showed that inclusion of FBs either fresh or ensiled at a level of 30% of the DM, resulted in a reduction of in vitro methane production and changing the ruminal fermentation pathway toward more propionate production, which considers non-methanogenic H^+ sink (Wang et al., 2018). Many studies, both in vitro and in vivo have shown that the ensiled or dried GP can reduce CH_4 production (Belibasakis et al., 1996; Pellikaan et al., 2011), and affect the rumen microbial profile, and methanogenic archaea (Biscarini et al., 2018). Moate et al. (2014) revealed that feeding of dried or ensiled GP to dairy cows resulted in a 20 % decrease in CH_4 emissions and a 23% decrease in CH_4 yield. To the best of the author`s knowledge, this is the first study to document that the PS addition can result in a reduction in enteric CH_4 emissions. These findings raise the question about the way by which the FBs inclusion resulted in methane reduction. In contrast with the prescribed hypothesis, ensiling did not affect the tannin content and that was contrary to the finding of Ott et al. (2005) who reported a reduction in CT content with the ensiling process. Due to the lack of available data, the result could not confirm that the ensiling did not change the chemical structure of the tannin. However, a recent study (Fitri et al., 2020) revealed that the ensiling resulted in the insolubilization of PS tannin reducing its antimethanogenic effect, and that was agreeing with the current result. On the other hand, the chemical composition of the inoculated

material played the main role. The ensiling process resulted in carbohydrate profile changing due to lactic acid bacteria, (e.g., *Lactobacillus* species), which utilized NFC to produce lactate and acetate (Mauro et al., 2013). This resulted in a decrease in methane production. Several explanations for this scenario are possible. First, propionate (H^+ sink) is produced due to the fermentation of silage lactic acid in the rumen by lactate-utilizing bacteria belonging to *Veillonellaceae* family such as *Selenomonas ruminantium*, *Megasphaera elsdenii,* and *Veillonella parvula* (Dawson et al., 1997; Russell and Wallace, 1997). Second, the compositional changes resulted in a significant increase in *Fibrobacter succinogenes* gene copies. *Fibrobacter succinogenes* is known as the primary cellulolytic organism, and it is a non-H_2-producing species. When the main fibrolytic species was non-H_2-producing, methane emission decreased significantly without impairing fiber degradation in the rumen (Chaucheyras-Durand et al., 2010). Third, silage inoculant maybe played a part in reducing methane, the LAB as silage inoculant or its metabolites may be directly inhibited the methanogen activity (Doyle et al., 2019). Collectively the inclusion of ensiled FBs at 30% DM could decrease the methane emission, particularly when used in a forage-based diet. In Chapter III, when the effect of the addition of PS in the fresh or ensiled form with dry absorbents mixture (KP and WB) on the rumen fermentation parameters was evaluated, the decrease in in vitro methane fermentation was also fetched by the PS even when absorbents were added. In contrast to the result of the previous in vitro cultivation (Chapter II), the abundance of *Fibrobacter* decreased in the culture containing ensiled PS with absorbents. This result discloses that the difference between the ensiled materials especially with the addition of the absorbent can affect the rumen microbial profile differently.

To ensure the possibility of application of the PSS in the animal diet, the acceptability by the animal was required to be evaluated. Silages with good fermentation quality not usually increase the silage intake; in fact, many factors are controlling the voluntary intake, such as the silage type and

physiological state of the animal. However, there is a strong indication that well-fermented silages (grass or whole crop) either treated or not with LAB inoculum would be more palatable for animals as when compared to the poorly-fermented ones (Okine, 2007). The agricultural by-product silages differ widely from the grass or whole crop silages in the regard of the palatability. Persimmon skin, in addition to its high moisture content, has astringent sensation in the mouth when eaten due to the tannin content. Tannins may have a determinant effect on voluntary intake of PSS, and animal ingestive behavior (Lamy et al., 2011). However, ensiling may offer opportunities to tannins to bind with other plant constitutes or fermentation end products instead of binding to the oral protein, causing the astringent effect (Scharenberg et al., 2007); the soluble tannin in astringent PS can be insolubilized by alcohol and carbon dioxide (Yamada et al., 2002). That may add another elucidation to the similar feed intake among groups in the current study. Since there were no previous studies on the provision of PSS on ruminants, a preliminary test was conducted, and 25 % of DM was an acceptable level of inclusion. Followed by a feeding trial conducted to evaluate the effect of partial substitution (20%) of DM content by PSS on palatability and ingestive behavior of sheep. The level of the inclusion was lower than that tested in the in vitro experiment and preliminary trial to avoid any health problem for the live animal. The results showed that a PSS-containing diet was acceptable to the sheep. The PSS-fed group spent a shorter time in eating compared to that in the control. The eating rate of the meal is well-thought-out as a good measure of appetite, and palatability is identified as the result of the physical and chemical characteristics that induce appetite (Scharenberg et al., 2007). Although the good palatability appeared in this experiment, care should be taken to avoid the accidental problems could happen due to the fast eating by offering the PSS with bulky roughage. The effect of the inclusion of the PSS on the ruminal parameters of dry ewes did not show any significant difference between groups except for the high butyrate proportion in the PSS group. The discrepancy between

the in vitro and in vivo regarding the VFA profile may be due to the low inclusion rate or due to long term microbial adaptation, so it essential to confirm the endurance of the antimethanogenic effect in vivo. The better digestibility that observed with PSS inclusion attributed to the ensiling process, *L.buchneri i*noculum can enhance animal nutrition and performance by various pathways. Firstly, through the production of some metabolites like bacteriocin, which may affect rumen bacteria (Basso et al., 2018). Secondly, having a direct-fed microbial or a probiotic effect when fed to the animal (Weinberg et al., 2007). Finally, this organism may produce an active metabolite in silage that alters it during storage, thereby improving its nutritive value by enhancing the digestibility of silage constituents (Nsereko et al., 2008). Some studies determined that *L. buchneri* produces a ferulate-esterase enzyme that enhances the degradability of the cell wall part, which in consequence, increases the release of soluble carbohydrates for fermentation during the ensiling or to rumen microorganism (De Oliveira et al., 2016). Therefore, the ensiling of FBs with inoculum and dry absorbent not only overcome the pre ensiling deficits in PS but also may motivate its digestibility, nutritional value, and methane inhibitory effect. Further exploration about the effect of the silage inoculum on rumen kinetics is highly reasonable.

The greenhouse gases emitted from the livestock production system are not only limited to the enteric methane but it includes all the GHG that associated with the animal product along with its supply chain starting with feed production, transportation, disposal, and ending with the processing and transportation of the end products. Although the enteric methane represents the largest single contributor to the sector's emissions with 39.1%, the use of manure and synthetic fertilizers for forage and feed production, processing, and transport are the most important contributors of GHG emissions related to the livestock sector (Rojas-Downing et al., 2017). These account to 45% of global livestock anthropogenic GHG emissions, involving mainly CO_2 and N_2O. Long-distance shipping is the major

GHG source in this group, for instance, high amounts of soybean are transported for long distances to be used as feedstuff (Steinfeld et al., 2006). Therefore, using of locally available PSM-rich fruits by-products besides being cheap feed resource, it can be an integral part of the GHG mitigation, by decreasing the carbon footprints from the importation of the feed sources, relieve the environmental load that can result from its decomposition, and finally, it can decrease the enteric methane from ruminant. The current study revealed to potentiality of the ensiled FBs as enteric methane mitigant, however, more research is needed to estimate the approximate GHG emissions reduction related to using the local FBs.

General Conclusion

The opportunity to use different FBs silages in ruminant feed was investigated in this thesis.

- Ensiling of FBs ensured long-term preservation and affected rumen fermentation, especially when it was included in a forage-based feed.
- Ensiled FBs could have direct or indirect effects on the reduction of methanogenesis.
- The compositional changes in the fibrous fraction during ensiling played a notable role in methane mitigation.
- Tannins in ensiled FBs seemed to have a little contribution to reduce in vitro ruminal methane.
- Improving PSS preservation quality and minimize the effluent flows could be achieved by using bacterial inoculant and dry absorbents.
- The inclusion of ensiled PS with the absorbents affects rumen fermentation patterns and the degree of methane generation in vitro.
- Incorporation of the PSS in sheep rations at level 20% of the DM showed suitable palatability and increased DM and NDF digestibility.

Suggestions for future research

In vitro techniques could inevitably offer a rapid evaluation method for methane mitigation potential of FBs as shown in Chapters II and III. However, further steps are still warranted such as in vivo studies to evaluate FBs as methane mitigants. Moreover, long-term feeding trials in different productive ruminants (growing and dairy) are required. In addition to that, this study did not evaluate the other effect of using FBs on the total GHG emission, hence further assessments are required to accomplish the establishment of this integrative strategy of the greenhouse gas mitigation.

References

Abarghuei, M.J., Rouzbehan, Y., Alipour, D., 2010. The influence of the grape pomace on the ruminal parameters of sheep. Livest. Sci. 132, 73–79. https://doi.org/10.1016/j.livsci.2010.05.002

Aboagye, I.A., Beauchemin, K.A., 2019. Potential of molecular weight and structure of tannins to reduce methane emissions from ruminants: A Review. Animals 9, 856. https://doi.org/10.3390/ani9110856

Abrar, A., Kondo, M., Kitamura, T., Ban-Tokuda, T., Matsui, H., 2016. Effect of supplementation of rice bran and fumarate alone or in combination on in vitro rumen fermentation, methanogenesis and methanogens. Anim. Sci. J. 87, 398–404. https://doi.org/10.1111/asj.12431

AFFRCS (Agriculture Forestry and Fisheries Research Council Secretariat), 1996. Equations to predict energy requirements. in Japanese Feeding Standard for Sheep; AFFRCS, Ed.; Japan Livestock Indust, Tokyo, Japan.

Aguerre, M.J., Capozzolo, M.C., Lencioni, P., Cabral, C., Wattiaux, M.A., 2016. Effect of quebracho-chestnut tannin extracts at 2 dietary crude protein levels on performance, rumen fermentation, and nitrogen partitioning in dairy cows. J. Dairy Sci. 99, 4476–4486. https://doi.org/10.3168/jds.2015-10745

Alejandro, Q.B.J., 1991. [Utilization of dry beet pulp, hay and wheat bran as absorbent additives on cutted silage]. [Spanish].

Alipour, D., Rouzbehan, Y., 2007. Effects of ensiling grape pomace and addition of polyethylene glycol on in vitro gas production and microbial biomass yield. Anim. Feed Sci. Technol. 137, 138–149. https://doi.org/10.1016/j.anifeedsci.2006.09.020

AOAC (Association of Official Analytical Chemists), 1990. Official Methods of analysis, 15th Ed. ed. Arlington, VA,USA.

Ashbell, G., Pahlow, G., Dinter, B., Weinberg, Z.G., 1987. Dynamics of orange peel fermentation during ensilage. J. Appl. Bacteriol. 63, 275–279. https://doi.org/10.1111/j.1365-2672.1987.tb02703.x

Basso, F.C., Rabelo, C.H.S., Lara, E.C., Siqueira, G.R., Reis, R.A., 2018. Effects of *Lactobacillus buchneri* NCIMB 40788 and forage: Concentrate ratio on the growth performance of finishing feedlot lambs fed maize silage. Anim. Feed Sci. Technol. 244, 104–115. https://doi.org/10.1016/j.anifeedsci.2018.08.008

Belibasakis, N.G., Progia, E., Papaioannou, A.., Skulidis, G., 1996. Effects of ensiled wet grape marc

on milk production. milk composition and blood components in dairy cows. agris.fao.org.

Ben Salem, H., Saghrouni, L., Nefzaoui, A., 2005. Attempts to deactivate tannins in fodder shrubs with physical and chemical treatments, in: Animal Feed Science and Technology. pp. 109–121. https://doi.org/10.1016/j.anifeedsci.2005.04.009

Benchaar, C., 2020. Feeding oregano oil and its main component carvacrol does not affect ruminal fermentation, nutrient utilization, methane emissions, milk production, or milk fatty acid composition of dairy cows. J. Dairy Sci. 103, 1516–1527. https://doi.org/10.3168/jds.2019-17230

Bhat, T.K., Singh, B., Sharma, O.P., 1998. Microbial degradation of tannins - A current perspectiveBhat, T. K., B. Singh, and O. P. Sharma. 1998. Microbial degradation of tannins - A current perspective. Biodegradation 9:343–357. Biodegradation 9, 343–357. https://doi.org/10.1023/A:1008397506963

Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O., Kurihara, M., 2009. Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J. Dairy Sci. 92, 5512–5522. https://doi.org/10.3168/jds.2008-1441

Biscarini, F., Palazzo, F., Castellani, F., Masetti, G., Grotta, L., Cichelli, A., Martino, G., 2018. Rumen microbiome in dairy calves fed copper and grape-pomace dietary supplementations: Composition and predicted functional profile. PLoS One 13. https://doi.org/10.1371/journal.pone.0205670

Bordiga, M., 2016. Valorization of wine making by-products. CRC press, Oca Raton, USA.

Brooker, J.D., O'Donovan, L.A., Skene, I., Clarke, K., Blackall, L., Muslera, P., 1994. *Streptococcus caprinus* sp.nov., a tannin-resistant ruminal bacterium from feral goats. Lett. Appl. Microbiol. 18, 313–318. https://doi.org/10.1111/j.1472-765X.1994.tb00877.x

Bryszak, M., Szumacher-Strabel, M., El-Sherbiny, M., Stochmal, A., Oleszek, W., Roj, E., Patra, A.K., Cieslak, A., 2019. Effects of berry seed residues on ruminal fermentation, methane concentration, milk production, and fatty acid proportions in the rumen and milk of dairy cows. J. Dairy Sci. 102, 1257–1273. https://doi.org/10.3168/jds.2018-15322

Cao, Y., Takahashi, T., Horiguchi, K. ich, Yoshida, N., Cai, Y., 2010a. Methane emissions from sheep fed fermented or non-fermented total mixed ration containing whole-crop rice and rice bran. Anim. Feed Sci. Technol. 157, 72–78. https://doi.org/10.1016/j.anifeedsci.2010.02.004

Cao, Y., Takahashi, T., Horiguchi, K., Yoshida, N., 2010b. Effect of adding lactic acid bacteria and molasses on fermentation quality and in vitro ruminal digestion of total mixed ration silage

prepared with whole crop rice. Grassl. Sci. 56, 19–25. https://doi.org/10.1111/j.1744-697X.2009.00168.x

Cao, Y., Takahashi, T., Horiguchi, K.I., Yoshida, N., Zhou, D., 2012. In vitro ruminal dry matter digestibility and methane production of fermented total mixed ration containing whole-crop rice and rice bran. Grassl. Sci. 58, 133–139. https://doi.org/10.1111/j.1744-697X.2012.00254.x

Carulla, J.E., Kreuzer, M., Machmüller, A., Hess, H.D., 2005. Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Aust. J. Agric. Res. 56, 961. https://doi.org/10.1071/AR05022

Chao, W., Uyeno, Y., Jayanegara, A., Kondo, M., Ban-Tokuda, T., Matsui, H., 2016. Changes in in vitro rumen fermentation characteristics of different compositions of total mixed rations (TMR) and the ensiled TMRs. Adv. Anim. Vet. Sci. 4, 178–182. https://doi.org/10.14737/JOURNAL.AAVS/2016/4.4.178.182

Chaucheyras-Durand, F., Masséglia, S., Fonty, G., Forano, E., 2010. Influence of the composition of the cellulolytic flora on the development of hydrogenotrophic microorganisms, hydrogen utilization, and methane production in the rumens of gnotobiotically reared lambs. Appl. Environ. Microbiol. 76, 7931–7937. https://doi.org/10.1128/AEM.01784-10

Chikwanha, O.C., Muchenje, V., Nolte, J.E., Dugan, M.E.R., Mapiye, C., 2019. Grape pomace (*itis vinifera L. cv. PinotageV*) supplementation in lamb diets: Effects on growth performance, carcass and meat quality. Meat Sci. 147, 6–12. https://doi.org/10.1016/j.meatsci.2018.08.017

Cottle, D.J., Nolan, J. V., Wiedemann, S.G., 2011. Ruminant enteric methane mitigation: A review. Anim. Prod. Sci. 51, 491–514. https://doi.org/10.1071/AN10163

Dawson, K.A., Rasmussen, M.A., Allison, M.J., 1997. Digestive disorders and nutritional toxicity, in: The Rumen Microbial Ecosystem. Springer Netherlands, pp. 633–660. https://doi.org/10.1007/978-94-009-1453-7_14

De Oliveira, J.S., Santos, E.M., Dos Santos, A.P.M., 2016. Intake and digestibility of silages, in: Advances in Silage Production and Utilization. InTech, Rijeka, Croatia, p. 101. https://doi.org/10.5772/65280

Dehority, B., 2003a. Numbers, factors affecting the population and distribution of rumen bacteria, in: Rumen Microbiology. Nottingham University Press, pp. 265–294.

Dehority, B., 2003b. Cellulose digesting rumen bacteria., in: Rumen Microbiology. Nottingham University Press, pp. 177–208.

Denman, S.E., Tomkins, N.W., McSweeney, C.S., 2007. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiol. Ecol. 62, 313–322. https://doi.org/10.1111/j.1574-6941.2007.00394.x

Doyle, N., Mbandlwa, P., Kelly, W.J., Attwood, G., Li, Y., Ross, R.P., Stanton, C., Leahy, S., 2019. Use of lactic acid bacteria to reduce methane production in ruminants, a critical review. Front. Microbiol. 10, 2207. https://doi.org/10.3389/fmicb.2019.02207

Eckard, R.J., Grainger, C., de Klein, C.A.M., 2010. Options for the abatement of methane and nitrous oxide from ruminant production: a review. Livest. Sci. 130, 47–56. https://doi.org/10.1016/j.livsci.2010.02.010

European Union, 2003. Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on additives for use in animal nutrition.

FAO (Food and Agriculture Organization of the United Nations), 2017. FAOSTAT. Rome, Italy.

Filya, I., 2003. The effect of *Lactobacillus buchneri* and *Lactobacillus plantarum* on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. J. Dairy Sci. 86, 3575–3581. https://doi.org/10.3168/jds.S0022-0302(03)73963-0

Fitri, A., Obitsu, T., Sugino, T., Jayanegara, A., 2020. Ensiling of total mixed ration containing persimmon peel: Evaluation of chemical composition and in vitro rumen fermentation profiles. Anim. Sci. J. 91. https://doi.org/10.1111/asj.13403

Fransen, S.C., Strubi, F.J., 1998. Relationships among absorbents on the reduction of grass silage effluent and silage quality. J. Dairy Sci. 81, 2633–2644. https://doi.org/10.3168/jds.S0022-0302(98)75821-7

Gandra, J.R., Oliveira, E.R., Gandra, E.R. de S., Takiya, C.S., Goes, R.H.T.B., Oliveira, K.M.P., Silveira, K.A., Araki, H.M.C., Orbach, N.D., Vasquez, D.N., 2017. Inoculation of *Lactobacillus buchneri* alone or with *Bacillus subtilis* and total losses, aerobic stability, and microbiological quality of sunflower silage. J. Appl. Anim. Res. 45, 609–614. https://doi.org/10.1080/09712119.2016.1249874

Gerber, P., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., 2013. Tackling climate change through livestock: a global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations (FAO).2013.

Goel, G., Makkar, H.P.S., 2012. Methane mitigation from ruminants using tannins and saponins. Trop.

Anim. Health Prod. 44, 729–739. https://doi.org/10.1007/s11250-011-9966-2

Goel, G., Puniya, A.K., Aguilar, C.N., Singh, K., 2005. Interaction of gut microflora with tannins in feeds. Naturwissenschaften. https://doi.org/10.1007/s00114-005-0040-7

Goeser, J.P., Heuer, C.R., Crump, P.M., 2015. Forage fermentation product measures are related to dry matter loss through meta-analysis. Prof. Anim. Sci. 31, 137–145. https://doi.org/10.15232/pas.2014-01356

Harborne, J.B., 2001. Twenty-five years of chemical ecology. Nat. Prod. Rep. 18, 361–379. https://doi.org/10.1039/b005311m

Hassan, Y.I., Kosir, V., Yin, X., Ross, K., Diarra, M.S., 2019. Grape pomace as a promising antimicrobial alternative in feed: a critical review. J. Agric. Food Chem. 67, 9705–9718. https://doi.org/10.1021/acs.jafc.9b02861

Herdt, T.H., 1988. Fuel homeostasis in the ruminant. Vet. Clin. North Am. Food Anim. Pract. 4, 213–231. https://doi.org/10.1016/S0749-0720(15)31045-8

Hiramori, C., Uyeno, Y., Koh, K., 2015. Effects of lactic acid bacteria on fermented apple pomace (FAP) production and inclusion of FAP in medium for bunashimeji (*Hypsizygus marmoreus*) cultivation. Mushroom Sci. Biotechnol. 23, 120–124. https://doi.org/10.24465/msb.23.3_120

Holzer, M., Mayrhuber, E., Danner, H., Braun, R., 2003. The role of *Lactobacillus buchneri* in forage preservation. Trends Biotechnol. 21, 282–287. https://doi.org/10.1016/S0167-7799(03)00106-9

Hook, S.E., Northwood, K.S., Wright, A.D.G., McBride, B.W., 2009. Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the lactating dairy cow. Appl. Environ. Microbiol. 75, 374–380. https://doi.org/10.1128/AEM.01672-08

Hristov, A.N., Ott, T., Tricarico, J., Rotz, A., Waghorn, G., Adesogan, A., Dijkstra, J., Montes, F., Oh, J., Kebreab, E., Oosting, S.J., Gerber, P.J., Henderson, B., Makkar, H.P.S., Firkins, J.L., 2013. Special topics-Mitigation of methane and nitrous oxide emissions from animal operations: III. A review of animal management mitigation options. J. Anim. Sci. 91, 5095–5113. https://doi.org/10.2527/jas.2013-6585

Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160, 1–22. https://doi.org/10.1016/j.anifeedsci.2010.07.002

Jayanegara, A., Togtokhbayar, N., Makkar, H.P.S., Becker, K., 2009. Tannins determined by various

methods as predictors of methane production reduction potential of plants by an in vitro rumen fermentation system. Anim. Feed Sci. Technol. 150, 230–237. https://doi.org/10.1016/j.anifeedsci.2008.10.011

JETRO (Japan External Trade Organization), 2016. Export Environment Survey (Fruit in South Korea).

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483–2492. https://doi.org/10.2527/1995.7382483x

Jones, G.A., McAllister, T.A., Muir, A.D., Cheng, K.J., 1994. Effects of sainfoin (*Onobrychis viciifolia Scop.*) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Appl. Environ. Microbiol.

Jones, R., Jones, D.I.H., 1996. The effect of in-silo effluent absorbents on effluent production and silage quality. J. Agric. Eng. Res. 64, 173–186. https://doi.org/10.1006/jaer.1996.0059

Jonsson, A., Pahlow, G., 1984. Systematic classification and biochemical characterization of yeast growing in grass silage inoculated with lactobacillus cultures. Anim. Res. Dev. 20, 7–22.

Kafantaris, I., Kotsampasi, B., Christodoulou, V., Makri, S., Stagos, D., Gerasopoulos, K., Petrotos, K., Goulas, P., Kouretas, D., 2018. Effects of dietary grape pomace supplementation on performance, carcass traits and meat quality of lambs. In Vivo (Brooklyn). 32, 807. https://doi.org/10.21873/INVIVO.112311

Kafantaris, I., Kotsampasi, B., Christodoulou, V., Kokka, E., Kouka, P., Terzopoulou, Z., Gerasopoulos, K., Stagos, D., Mitsagga, C., Giavasis, I., Makri, S., Petrotos, K., Kouretas, D., 2017. Grape pomace improves antioxidant capacity and faecal microflora of lambs. J. Anim. Physiol. Anim. Nutr. (Berl). 101, e108–e121. https://doi.org/10.1111/jpn.12569

Kasapidou, E., Sossidou, E., Mitlianga, P., 2015. Fruit and vegetable co-products as functional feed ingredients in farm animal nutrition for improved product quality. Agriculture 5, 1–15.

Kido, K., Tejima, S., Haramiishi, M., Uyeno, Y., Ide, Y., Kurosu, K., Kushibiki, S., 2019. Provision of beta- glucan prebiotics (cellooligosaccharides and kraft pulp) to calves from pre- to post-weaning period on pasture. Anim. Sci. J. 90, 1537–1543. https://doi.org/10.1111/asj.13299

Kim, H.Y., Song, Y.M., Kang, Y.S., Kim, C.H., Lee, S.D., Chowdappa, R., Ha, J.H., Kang, S.M., 2006. The effect of fermented persimmon shell diet supplementation on the growth performance and blood parameters in finishing pigs. Anim. Sci. J. 77, 314–319. https://doi.org/10.1111/j.1740-0929.2006.00354.x

Kim, S.B., Kim, D.H., Lee, H.J., Amanullah, S.M., Kim, S.C., 2013. Effects of fermented persimmon extract supplements on chemical composition and fermentation characteristics of barley silage. Anim. Sci. J. 84, 403–408. https://doi.org/10.1111/asj.12023

Kleinschmit, D.H., Schmidt, R.J., Kung, L., 2005. The effects of various antifungal additives on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 88, 2130–2139. https://doi.org/10.3168/jds.S0022-0302(05)72889-7

Kondo, M., Jayanegara, A., Uyeno, Y., Matsui, H., 2015. Variation of tannin contents in selected agro-industrial by-products and their biological activity in precipitating protein. Adv. Anim. Vet. Sci. 4, 66–70. https://doi.org/10.14737/journal.aavs/2016/4.2.66.70

Kordi, M., Naserian, A.A., 2012. Influence of wheat bran as a silage additive on chemical composition, in situ degradability and in vitro gas production of citrus pulp silage. African J. Biotechnol. 11, 12669–12674. https://doi.org/10.5897/AJB12.767

Krooneman, J., Faber, F., Alderkamp, A.C., Oude Elferink, S.J.H.W., Driehuis, F., Cleenwerck, I., Swings, J., Gottschal, J.C., Vancanneyt, M., 2002. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. Int. J. Syst. Evol. Microbiol. 52, 639–646. https://doi.org/10.1099/00207713-52-2-639

Krueger, W.K., Gutierrez-Bañuelos, H., Carstens, G.E., Min, B.R., Pinchak, W.E., Gomez, R.R., Anderson, R.C., Krueger, N.A., Forbes, T.D.A., 2010. Effects of dietary tannin source on performance, feed efficiency, ruminal fermentation, and carcass and non-carcass traits in steers fed a high-grain diet. Anim. Feed Sci. Technol. 159, 1–9. https://doi.org/10.1016/j.anifeedsci.2010.05.003

Krumholz, L.R., Bryant, M.P., 1986. *Eubacterium oxidoreducens* sp. nov. requiring H 2 or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. Arch. Microbiol. 144, 8–14. https://doi.org/10.1007/BF00454948

Lamy, E., Rawel, H., Schweigert, F.J., Silva, F.C.E., Ferreira, A., Costa, A.R., Antunes, C., Almeida, A.M., Coelho, A.V., Sales-Baptista, E., 2011. The effect of tannins on mediterranean ruminant ingestive behavior: The role of the oral cavity. Molecules 16, 2766–2784. https://doi.org/10.3390/molecules16042766

Lee, Y.A., Cho, E.J., Yokozawa, T., 2008. Protective effect of persimmon (Diospyros kaki) peel proanthocyanidin against oxidative damage under H_2O_2-induced cellular senescence. Biol. Pharm. Bull. 31, 1265–1269. https://doi.org/10.1248/bpb.31.1265

Lesschaeve, I., Noble, A.C., 2005. Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. Am. J. Clin. Nutr. 81, 330s-335s. https://doi.org/10.1093/ajcn/81.1.330s

Lettat, A., Hassanat, F., Benchaar, C., 2013. Corn silage in dairy cow diets to reduce ruminal methanogenesis: Effects on the rumen metabolically active microbial communities. J. Dairy Sci. 96, 5237–5248. https://doi.org/10.3168/jds.2012-6481

Lu, Y., Yeap Foo, L., 1999. The polyphenol constituents of grape pomace. Food Chem. 65, 1–8. https://doi.org/10.1016/S0308-8146(98)00245-3

Maeda, Y., Nishimura, K., Kurosu, K., Mizuguchi, H., Sato, S., Terada, F., Kushibiki, S., 2019. Effect of feeding wood kraft pulp on the growth performance, feed digestibility, blood components, and rumen fermentation in Japanese Black fattening steers. Anim. Sci. J. 90, 523–532. https://doi.org/10.1111/asj.13182

Makkar, H.P.S., 2003a. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds, in: Small Ruminant Research. Elsevier, pp. 241–256. https://doi.org/10.1016/S0921-4488(03)00142-1

Makkar, H.P.S., 2003b. Quantification of tannins in tree and shrub foliage, quantification of tannins in tree and shrub foliage. Springer Netherlands. https://doi.org/10.1007/978-94-017-0273-7

Makkar, H.P.S., Blümmel, M., Becker, K., 1995. In vitro effects of and interactions between tannins and saponins and fate of tannins in the rumen. J. Sci. Food Agric. 69, 481–493. https://doi.org/10.1002/jsfa.2740690413

Malik, P.K., Kolte, A.P., Baruah, L., Saravanan, M., Bakshi, B., Bhatta, R., 2017. Enteric methane mitigation in sheep through leaves of selected tanniniferous tropical tree species. Livest. Sci. 200, 29–34. https://doi.org/10.1016/j.livsci.2017.04.001

Massaro Junior, F.L., Bumbieris Junior, V.H., Zanin, E., Silva, L. das D.F., Galbeiro, S., Pereira, E.S., Neumann, M., Mizubuti, I.Y., 2020. Effect of storage time and use of additives on the quality of grape pomace silages. J. Food Process. Preserv. e14373. https://doi.org/10.1111/jfpp.14373

Mauro, E., da Silva, T.C., Oliveira Macedo, C.H., Sena, F., 2013. Lactic acid bacteria in tropical grass silages, in: Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes. InTech, pp. 335–362. https://doi.org/10.5772/50703

McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agric. 48, 7–13. https://doi.org/10.1071/EA07218

McDonald, P., Henderson, N., Heron, S., 1991. The biochemistry of silage, 2nd ed. Chalcombe publications, Aberyswyth, UK.

McSweeney, C.S., Palmer, B., McNeill, D.M., Krause, D.O., 2001. Microbial interactions with tannins: Nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91, 83–93. https://doi.org/10.1016/S0377-8401(01)00232-2

Moate, P.J., Deighton, M.H., Williams, S.R.O., Pryce, J.E., Hayes, B.J., Jacobs, J.L., Eckard, R.J., Hannah, M.C., Wales, W.J., 2016. Reducing the carbon footprint of Australian milk production by mitigation of enteric methane emissions. Anim. Prod. Sci. 56, 1017–1034. https://doi.org/10.1071/AN15222

Moate, P.J., Williams, S.R.O., Torok, V.A., Hannah, M.C., Ribaux, B.E., Tavendale, M.H., Eckard, R.J., Jacobs, J.L., Auldist, M.J., Wales, W.J., 2014. Grape marc reduces methane emissions when fed to dairy cows. J. Dairy Sci. 97, 5073–5087. https://doi.org/10.3168/jds.2013-7588

Mokhtarpour, A., Naserian, A.A., Pourmollae, F., Ghaffari, M.H., 2016. Effect of treating alfalfa silage with pistachio by-products extract on Saanen dairy goats performance and microbial nitrogen synthesis. J. Anim. Physiol. Anim. Nutr. (Berl). 100, 758–767. https://doi.org/10.1111/jpn.12384

Molan, A.L., Attwood, G.T., Min, B.R., McNabb, W.C., 2001. The Effect of condensed tannins from *Lotus Pedunculatus* and *Lotus Corniculatus* on the growth of proteolytic rumen bacteria in vitro and their possible mode of action. Can. J. Microbiol. 47, 626–633. https://doi.org/10.1139/w01-060

Moran, J., 2005. Tropical dairy farming: feeding management for small holder dairy farmers in the humid tropics.

Muck, R., Nadeau, E., McAllister, T., Contreras-Govea, F., Santos, M., Kung Jr, L., 2018. Silage review: Recent advances and future uses of silage additives. J. Dairy Sci. 101, 3980–4000. https://doi.org/10.3168/jds.2017-13839

NARO (National Agriculture and Food Research Organization), 2009. Standard Tables of Feed Composition in Japan, Japan Livestock Industry Association. Tokyo, Japan.

Nelson, K.E., Pell, A.N., Schofield, P., Zinder, S., 1995. Isolation and characterization of an anaerobic ruminal bacterium capable of degrading hydrolyzable tannins. Appl. Environ. Microbiol. 61, 3293–8.

Nelson, K.E., Thonney, M.L., Woolston, T.K., Zinder, S.H., Pell, A.N., 1998. Phenotypic and

phylogenetic characterization of ruminal tannin-tolerant bacteria. Appl. Environ. Microbiol. 64, 3824–30.

Nishimura, K., Kurosu, K., Terada, F., Mizuguchi, H., Sato, S., Kushibiki, S., 2019. Effect of wood kraft pulp feed on digestibility, ruminal characteristics, and milk production performance in lactating dairy cows. Anim. Sci. J. 90, 189–195. https://doi.org/10.1111/asj.13131

Nishino, N., Wada, H., Yoshida, M., Shiota, H., 2004. Microbial counts, fermentation products, and aerobic stability of whole crop corn and a total mixed ration ensiled with and without inoculation of Lactobacillus casei or Lactobacillus buchneri. J. Dairy Sci. 87, 2563–70. https://doi.org/10.3168/jds.S0022-0302(04)73381-0

Nsereko, V.L., Smiley, B.K., Rutherford, W.M., Spielbauer, A., Forrester, K.J., Hettinger, G.H., Harman, E.K., Harman, B.R., 2008. Influence of inoculating forage with lactic acid bacterial strains that produce ferulate esterase on ensilage and ruminal degradation of fiber. Anim. Feed Sci. Technol. 145, 122–135. https://doi.org/10.1016/j.anifeedsci.2007.06.039

Nudda, A., Correddu, F., Marzano, A., Battacone, G., Nicolussi, P., Bonelli, P., Pulina, G., 2015. Effects of diets containing grape seed, linseed, or both on milk production traits, liver and kidney activities, and immunity of lactating dairy ewes. J. Dairy Sci. 98, 1157–1166. https://doi.org/10.3168/jds.2014-8659

Okine, A., Yimamu, A., Hanada, M., Izumita, M., Zunong, M., Okamoto, M., 2007. Ensiling characteristics of daikon (*Raphanus sativus*) by-product and its potential as an animal feed resource. Anim. Feed Sci. Technol. 136, 248–264. https://doi.org/10.1016/j.anifeedsci.2006.09.005

Okine, A.R.A., 2007. Improving the preservation quality of high moisture by-product feedstuffs by ensilage and use of additives. (Doctoral dissertation, Iwate University), Iwate, Japan.

Oladosu, Y., Rafii, M.Y., Abdullah, N., Magaji, U., Hussin, G., Ramli, A., Miah, G., 2016. Fermentation quality and additives: a case of rice straw silage. Biomed Res. Int. 2016, 7985167. https://doi.org/10.1155/2016/7985167

Ott, E., Aragón, A., Gabel, M., 2005. Ensiling of tannin-containing sorghum grain, in: Proceedings of the 14th International Silage Conference. pp. 3–6.

Özkul, H., Kiliç, A., Polat, M., 2011. Evaluation of mixtures of certain market wastes as silage. Asian-Australasian J. Anim. Sci. 24, 1243–1248. https://doi.org/10.5713/ajas.2011.10460

Patra, A.K., Saxena, J., 2011. Exploitation of dietary tannins to improve rumen metabolism and

ruminant nutrition. J. Sci. Food Agric. 91, 24–37. https://doi.org/10.1002/jsfa.4152

Patra, A.K., Saxena, J., 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71, 1198–1222. https://doi.org/10.1016/j.phytochem.2010.05.010

Pellikaan, W.F., Stringano, E., Leenaars, J., Bongers, D.J.G.M., Schuppen, S. van L. van, Plant, J., Mueller-Harvey, I., 2011. Evaluating effects of tannins on extent and rate of in vitro gas and CH4 production using an automated pressure evaluation system (APES). Anim. Feed Sci. Technol. 166–167, 377–390. https://doi.org/10.1016/j.anifeedsci.2011.04.072

Pereira, L.G.R., Machado, F.S., Campos, M.M., Júnior, R.G., Tomich, T.R., Reis, L.G., Coombs, C., 2015. Enteric methane mitigation strategies in ruminants: A review. Rev. Colomb. Ciencias Pecu. 28, 124–143. https://doi.org/10.17533/udea.rccp.v28n2a02

Razak, O.A., Masaaki, H., Yimamu, A., Meiji, O., 2012. Potential water retention capacity as a factor in silage effluent control: experiments with high moisture by-product feedstuffs. Asian-Australasian J. Anim. Sci. 25, 471–8. https://doi.org/10.5713/ajas.2011.11349

Reed, J.D., 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. J. Anim. Sci. 73, 1516–1528. https://doi.org/10.2527/1995.7351516x

Rojas-Downing, M.M., Nejadhashemi, A.P., Harrigan, T., Woznicki, S.A., 2017. Climate change and livestock: Impacts, adaptation, and mitigation. Clim. Risk Manag. https://doi.org/10.1016/j.crm.2017.02.001

Romero-Perez, A., Okine, E.K., McGinn, S.M., Guan, L.L., Oba, M., Duval, S.M., Kindermann, M., Beauchemin, K.A., 2015. Sustained reduction in methane production from long-term addition of 3-nitrooxypropanol to a beef cattle diet. J. Anim. Sci. 93, 1780–1791. https://doi.org/10.2527/jas.2014-8726

Russell, J., 2002. Rumen microbiology and its role in ruminant nutrition. Dept. of Microbiology Cornell University, Ithaca NY.

Russell, J.B., Rychlik, J.L., 2001. Factors that alter rumen microbial ecology. Science (80-.). 292, 1119–1122.

Russell, J.B., Wallace, R.J., 1997. Energy-yielding and energy-consuming reactions, in: The Rumen Microbial Ecosystem. Springer Netherlands, pp. 246–282. https://doi.org/10.1007/978-94-009-1453-7_6

Scharenberg, A., Arrigo, Y., Gutzwiller, A., Soliva, C.R., Wyss, U., Kreuzer, M., Dohme, F., 2007.

Palatability in sheep and in vitro nutritional value of dried and ensiled sainfoin (*Onobrychis viciifolia*) birdsfoot trefoil (*Lotus corniculatus*), and chicory (*Cichorium intybus*). Arch. Anim. Nutr. 61, 481–496. https://doi.org/10.1080/17450390701664355

Smith, A.H., Imlay, J.A., Mackie, R.I., 2003. Increasing the oxidative stress response allows *Escherichia coli* to overcome inhibitory effects of condensed tannins. Appl. Environ. Microbiol. 69, 3406–3411. https://doi.org/10.1128/AEM.69.6.3406-3411.2003

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., 2006. Livestock's long shadow: environmental issues and options.

Stevenson, D.M., Weimer, P.J., 2007. Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl. Microbiol. Biotechnol. 75, 165–174. https://doi.org/10.1007/s00253-006-0802-y

Tabacco, E., Righi, F., Quarantelli, A., Borreani, G., 2011. Dry matter and nutritional losses during aerobic deterioration of corn and sorghum silages as influenced by different lactic acid bacteria inocula. J. Dairy Sci. 94, 1409–19. https://doi.org/10.3168/jds.2010-3538

Ungerfeld, E.M., 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: A meta-analysis. Front. Microbiol. 6, 37. https://doi.org/10.3389/fmicb.2015.00037

Uyeno, Y., 2015. Selective inhibition of harmful rumen microbes, in: Rumen Microbiology: From Evolution to Revolution. Springer India, pp. 199–211. https://doi.org/10.1007/978-81-322-2401-3_14

Uyeno, Y., Chao, W., Jayanegara, A., Kondo, M., Ban-Tokuda, T., Matsui, H., 2016a. Increase in rumen fibrolytic bacteria and the improvement of fiber degradability of ensiled total mixed ration assessed by in vitro rumen culture. Adv. Anim. Vet. Sci. 4, 183–186. https://doi.org/10.14737/JOURNAL.AAVS/2016/4.4.183.186

Uyeno, Y., Konaka, R., Shirota, M., Kobayashi, S., 2016b. Ensiling fruit by-products with inoculum of lactic acid bacteria strains. Anim. Nutr. Feed Technol. 16, 515–519. https://doi.org/10.5958/0974-181X.2016.00041.X

Van Keulen, J., Young, B.A., 1977. Evaluation of Acid-Insoluble Ash as a Natural Marker in Ruminant Digestibility Studies. J. Anim. Sci. 44, 282–287. https://doi.org/10.2527/jas1977.442282x

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber,

and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Wambacq, E., Vanhoutte, I., Audenaert, K., De Gelder, L., Haesaert, G., 2016. Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: A review. J. Sci. Food Agric. 96, 2284–2302. https://doi.org/10.1002/jsfa.7565

Wang, K., Nan, X., Chu, K., Tong, J., Yang, L., Zheng, S., Zhao, G., Jiang, L., Xiong, B., 2018. Shifts of hydrogen metabolism from methanogenesis to propionate production in response to replacement of forage fiber with non-forage fiber sources in diets in vitro. Front. Microbiol. 9, 2764. https://doi.org/10.3389/fmicb.2018.02764

Weinberg, Z.G., Shatz, O., Chen, Y., Yosef, E., Nikbahat, M., Ben-Ghedalia, D., Miron, J., 2007. Effect of lactic acid bacteria inoculants on in vitro digestibility of wheat and corn silages. J. Dairy Sci. 90, 4754–4762. https://doi.org/10.3168/jds.2007-0176

Woolford, M.K., 1978. The problem of silage effluent. Herb. Abstr. 48, 397–403.

Yamada, M., Taira, S., Ohtsuki, M., Sato, A., Iwanami, H., Yakushiji, H., Wang, R., Yang, Y., Li, G., 2002. Varietal differences in the ease of astringency removal by carbon dioxide gas and ethanol vapor treatments among Oriental astringent persimmons of Japanese and Chinese origin. Sci. Hortic. (Amsterdam). 94, 63–72. https://doi.org/10.1016/S0304-4238(01)00367-3

Yaqub, S., Farooq, U., Shafi, A., Akram, K., Murtaza, M.A., Kausar, T., Siddique, F., 2016. Chemistry and functionality of bioactive compounds present in persimmon. J. Chem. 2016. https://doi.org/10.1155/2016/3424025

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