Evaluation of ginkgo fruit as a new feed additive candidate for ruminant animals

（反芻家畜における新規飼料添加物候補 ギンナン果肉の評価）

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Chapter 1. Research background and literature review

Rumen and its microbes

Ruminants belong to herbivorous animals, provide their products such as meat and milk to human. They consume a wide variety of feed sources represented by plant polysaccharides, although they do not produce and secrete digestive enzymes for degrading such plant components. Their stomach is composed of 4 compartments, namely rumen, reticulum, omasum and abomasum, forming a unique digestive system. Of these, the section rumen is a large bio-fermenter which is colonized by a diverse microbiota. Rumen microbes significantly contribute to digestion of plant materials, providing fibrolytic enzymes to their host animal. Rumen microbes are classified into several groups, bacteria (10^{10}-10^{11}/mL), archaea (10^{4}-10^{7}/mL), protozoa (10^{4}-10^{6}/mL), fungi (10^{3}-10^{6}/mL) and bacteriophage (>10^{7} plaque forming unit/mL) (Hespell, 1997; Morvan et al., 1996; Williams and Coleman, 1997; Orpin and Joblin, 1997; Ross et al., 2013).

Bacteria

Bacteria are known to be a major group to degrade plant materials due to their larger abundance and versatile metabolic properties in comparison with other microbes (Lee et al., 2000). Although most of rumen microbes have not been culturable and their roles have not been clear (Stiverson et al., 2011), culturable bacteria have provided comprehensive knowledge in ruminant nutrition for understanding and also improving feed utilization. Over the past 60 years, many species of rumen bacteria have been isolated and characterized, which led deeper understandings and insights concerning rumen microbes (Bryant, 1959; Krause et al., 2013). Those can be

summarized as follows.

Fibrobacter succinogenes, *Ruminococcus flavefaciens* and *Ruminoccocus albus* are recognized to be major rumen fibrolytic bacterial species, because of their higher cellulolytic activity than other species. These fibrolytic species possess a variety of chemical properties to degrade complex carbohydrate polymers (Koike and Kobayashi, 2009) that are composed by cellulose and hemicellulose as major components and other minor components (VanSoest, 1982),

Ruminococcus flavefaciens produces acetate, formate, H_2, CO_2, and succinate, while *Ruminocuccus albus* produces same products but ethanol instead of succinate. These species are commonly classified into Gram-positive bacteria and utilize cellulose and hemicellulose. *Fibrobacter succinogenes*, a Gram-negative bacterium, produces formate, acaetate and succinate. Cellulose is a preferred substrate for this species. Hemicellulose is not utilized, although this bacterium can hydrolyzes it.

Butyrivibrio fibrisolvens is a motile rod having various functions in the rumen. *B. fibrisolvens* produces butyrate, formate, lactate, acetate, ethanol, H_2 and CO_2. This species utilizes pentose, hexose, pectin, starch, xylans and hemicellulose. Initially, *Butyrivibrio fibrisolvens* was known to be a Gram negative bacterium, but later Cheng and Costerton (1977) revealed that this bacterium has cell wall structure belonging to Gram-positives. *Eubacterium ruminantium* is a non-motile Gram-positive short rod. This species utilizes maltodextrin and other sugars to produce formate, acetate, butyrate and lactate. Ammonia is essential for the growth of this species.

Prevotella species are non-motile Gram-negative rods, playing important roles in the rumen. They have a wide variety of functions (Bekele et al., 2010), and are present in the rumen

abundantly (42-60% to total bacterial 16S rRNA copies). Four species (*Prevotella rumonicola, Prevotella bryantii, Prevotella albensis* and *Prevotella brevis*) are known, but their population in total bacterial are not very high (2-4%) (Stevenson and Weimer, 2007). They can utilize a variety of substrates including starch, hemicellulose, pectin, hexose and protein. Formate, acetate, propionate and succinate are produced. Although, *Prevotella ruminicola* does not utilize crystalline cellulose, this species contributes to fiber degradation by synergistic action with *Fibrobacter succinogenes* (Osborne and Dehority, 1989).

Selenomonas ruminantium produces acetate, propionate, butyrate, succinate, lactate, H_2 and CO_2. Most strains utilize sugars, and some utilize starch (Prins, 1971). Although *Selenomonas ruminantium* does not directly utilize plant fiber, it stimulates fiber degradation when co-cultured with *Fibrobacter succinogens* (Sawanon et al., 2011). This bacterium is a Gram-negative crescent-rod having flagella, although it belongs to *Firmicutes*. Recently, Genus *Selonomonas* is re-classified into *Selenomonadaceae* family nov., *Selenomonadales* order emend., *Negativicutes* class nov. (Marchandin et al., 2010; Campbell et al., 2015).

Megasphaera elsdenii is a Gram-negative large coccus, producing acetate, propionate, butyrate, valerate, caproate, H_2 and CO_2. *Megasphaera elsdenii* ferments a variety of sugars and converts lactate to produce propionate and butyrate. About 80% of lactate turnover in dairy cattle rumen fed grain diet is performed by *Megasphaera elsdenii* (Counotte et al., 1983). Previously, this organism was classified as *Peptostreptococcus elsdenii*, but recently, due to morphological and genetic mismatch, *Megasphaera elsdenii* is reclassified into *Vellionellaceae* family, *Vellionellales* order nov., *Negativicutes* class nov. (Marchandin et al., 2010; Campbell et al., 2015).

Anaerovibrio lipolytia is a Gram-negative curved rod with a flagellum. *A. lipolytica* can hydrolyzes tri-glycerides and glycerol. *A. lipolytica* also utilizes a narrow range of substrates, fructose among sugars and limited amount of lactate. Major end-products are acetate, propionate and succinate. Genus *Anaerovibrio* is recently classified into *Acidaminococcaceae* family nov., *Acidaminococcales* order nov., *Negativicutes* class nov. (Marchandin et al., 2010; Campbell et al., 2015).

Streptococcus bovis, Gram-positive coccus, producing formate, acetate, lactate and ethanol, utilizes starch and sugars. Ammonia can be used as a nitrogen source. *S. bovis* grows faster in pure culture and as well as in the rumen at high level of soluble carbohydrate supplementation. When *S. bovis* is dominant in the rumen, lactate is accumulated and ruminal pH is drastically decreased. Because *S. bovis* is relatively tolerant to low pH than other rumen bacteria, this species becomes predominant under low pH condition, and then rumen lactate acidosis can occur.

Ruminobacter amylophilus, a Gram-negative bacterium formerly called as *Bacteroides amylophilus*. This bacterium utilizes maltose, dextrin or starch but not glucose. Ammonia is used as nitrogen source. Formate, acetate and succinate are produced. *Succinimonas amylolytica* is a Gram-negative rod. This species utilizes starch, and produces acetate, propionate and succinate. *Succinivibrio dextrinosolvens* is a Gram-negative helically-shaped bacterium. This species does not utilize starch, but do maltodextrins released from other bacteria that have amylase.

Treponema bryantii is commonly detectable in the rumen. This species has Gram-negative cell wall, spiral shape and flagella that make the bacterium highly motile. Their functions in the rumen are versatile. Formate, acetate and succinate are major end-products. This species

significantly contributes to plant fiber degradation because of considerable utilization of pectin and xylan that are associated with cellulose (Stanton and Canale-Parola, 1980). Therefore, a positive interaction with *Fibrobacter succinogenes* in fiber degradation has been reported (Stanton and Canale-Parola, 1980).

Chen and Russell (1989) isolated three species of rumen bacteria from medium enriched in Trypticase. Although these bacteria did not utilize carbohydrates, they deaminate amino acid rapidly and produce ammonia extensively. Later, these bacteria fermenting peptides and amino acids are recognized as *Peptostreptococcus anaerobius, Clostridium sticklandii* and *Clostridium aminophilum* by 16S rRNA sequencing (Krause and Russell, 1996). *Peptostreptococcus anaerobius* produces acetate and branched chain fatty acid, and *Clostridium sticklandii* produces acetate, branched fatty acid, butyrate and small amount of propionate, while *Clostridium aminophilum* produces acetate and butyrate. These species are currently termed as hyper ammonia producing bacteria in the rumen. Excessive amino acid degradation is recognized as an undesired function in the rumen, because high production of ammonia in the rumen is not beneficial for nitrogen economy. It is generally accepted that 5 mg ammonia-nitrogen/dL is critical level for maintaining microbial synthesis and higher levels can lead nitrogen loss (Griswold et al., 2003).

Methanogens

Enteric methane is produced by methanogenic archaea in gastrointestinal tract of ruminant animals during their feed utilization, which closely related to greenhouse gas emission and loss of energy in feed stuff (Hook et al., 2010). Methanogens have various cell morphology and each

group have unique characteristics to develop interactions in methane synthesis with protozoa and fungi (Vogels et al., 1980; Cheng et al., 2009). These are based on transfer of hydrogen produced by protozoa or fungi to methanogen.

Methanobacteriaceae belonging to order *Methanobacteriales* has Gram-positive type cell wall and variable morphology, and contains *Methanobacterium* and *Methanobrevibacter* genera relatively abundant in the rumen (Henderson et al., 2015). Methanogenic archaea in these two genera utilize similar substrate for methane production. *Methanobacterium formicicum, Methanobrevibacter smithii* and *Methanobrevibacter ruminantium* can use H_2, CO_2 and formate, while *Methanobacterim bryantii* and *Methanobrevibacter wolinii* are not able to use formate (Balch et al., 1979; Godsy, 1980; Miller et al., 1982; Miller and Lin, 2002). *Methanomicrobium mobile,* belonging to family Methanomicrobiaceae, is Gram-negative, rod-shaped and motile, utilizing H_2, CO_2 and formate as substrate (Balch et al., 1979). *Methanosarcina barkeri,* having coccoid-shaped Gram-positive cell without motility, possesses diverse pathways to produce methane with H_2, CO_2, formate, methanol, acetate and methylamine (Balch et al., 1979). *Methanosarcina* strains are also found in sewage and sediment (Maeder et al., 2006). *Methanomassilliicoccaceae* is a newly proposed group which belongs to *Methanomassilliicoccales* order nov. (Iino et al., 2013). Although knowledge in ecology of this group of methanogens is poor, attention is being paid to this group because of their contribution to methane production based on meta-transcriptomic analysis of cattle rumen (Poulsen et al., 2013).

Protozoa and fungi

Protozoa are much less abundant (10^4-10^6/mL) than bacteria (10^{10}-10^{11}/mL), although they represent 50% of microbial biomass in the rumen (Jouany and Ushida, 1999). They engulf bacteria and small protozoa (Belanche et al., 2014). They are classified into two group that are order *Trichostomatida*, family *Isotrichidae* utilizing various substrate, and order *Entodiniomorphida*, family *Ophryoscolecidae* utilizing soluble sugars (Jouany and Ushida, 1999). Gijzen et al. (1988) estimated that contribution of protozoa in fiber degradation is 19-28%. Dijkstra and Tamminga (1995) noted that protozoal contribution in NDF degradation is less significant than bacteria because their contribution is easily affected by diet factors such as feed intake and type of substrate. Protozoa compose a symbiotic relationship with methanogen, because, H_2 accumulation is inhibitory to protozoal metabolism, while H_2 is utilized by methanogen as a main substrate (Belanche et al., 2014). It has been estimated that methanogens associated with protozoa are responsible for 9-37% methane production (Newbold et al., 2015). *Methanobacteriaceae* is recognized as important symbionts of rumen protozoa (Sharp et al., 1998).

Rumen anaerobic fungi also contribute to fiber degradation, producing acetate, formate, lactate, ethanol, H_2 and CO_2 (Cheng et al., 2009). Rumen anaerobic fungi are less abundant compared to bacteria, and their contribution in fiber degradation is less appreciated, although they showed positive interactions in fiber degradation with methanogens (Bauchop and Mounfort, 1981) and with *Fibrobacter succinogenes* (Bernalier et al., 1992) in co-culture study.

Rumen fermentation and its modulation

As the result of host-microbial interaction, various end-products such as short chain fatty acids (SCFAs), ammonia and gases are produced. SCFAs are major energy sources for ruminants, contributing up to 70% of energy requirement of cattle (Bergman, 1990). Rumen SCFAs are produced from various sources such as carbohydrate as a major substrate, dietary proteins, pentoses of nucleic acids, and glycerol of glycerophospholipids (Nafikov and Beitz, 2007). Most of dietary feed stuff rich in carbohydrate is fermented to acetate, propionate, butyrate as major SCFAs, and valerate and caproate as minor ones (Hungate, 1966). Explicit directions of rumen improvement may be involved in dietary treatment to meet specific purpose such as meat and milk quality (Calsamiglia et al., 2007) and use of antibiotics for microbial shift either to reduce enteric methane production relating to feed efficiency (Hook et al., 2010) or to minimize risk of digestive disorder like rumen acidosis (Golder et al., 2014).

Acetate is highly a lipogenic precursor in ruminants, and sites of lipogenesis are adipose tissue and mammary gland in lactating period (Vernon et al., 2001). Milk fat concentration is essentially affected by supply of acetate and butyrate (Emery, 1988). We can expect that dietary factors including roughage type, forage to concentrate ratio, carbohydrate composition of concentrates, lipids, feed intake, and meal frequency are important for manipulating concentration of milk fat (Sutton, 1989). Propionate is typically synthesized via fumarate reduction (Macy et al., 1978), and also via acrylate pathway which does not require succinate as an intermediate (Baldwin et al., 1962). Propionate serves to synthesize glucose via gluconeogenesis in the liver and kidney (Aschenbach et al., 2010). High level of plasma propionate is beneficial for body weight gain even when glucogenic precursors are sufficient (Jenkins and Thonney, 1988). Thus, propionate is an

important metabolite in the rumen. Therefore, enhancement of propionate has been one of the main targets in modulation of rumen fermentation. Often propionate enhancement is accompanied with methane reduction, another favorable change in fermentation product. Because both pathways to produce propionate and methane compete each other to metabolize hydrogen, propionate enhancement and methane reduction have been often attempted at the same time.

Strategies to improve rumen fermentation include dietary treatment (Beauchemin et al., 2008), ionophores (Guan et al., 2006), essential oils (Patra and Yu, 2012), vaccination (Wedlock et al., 2013) and defaunation (Morgavi et al., 2012). These are essentially related to alteration of metabolic hydrogen flow to a favorable way to generate propionate in the rumen (Ungerfeld, 2015). Control of metabolic hydrogen is consequently connected to reduce a wasteful product such as methane and to enhance useful product including propionate (Ungerfeld, 2015).

Ionophore antibiotics

Ionophore antibiotics represented by monensin have been used in many countries since those were approved in 1970s. Studies confirmed that ionophores are effective to modulation of rumen fermentation (Russell and Strobel, 1989). Ionophores decrease lactate concentration which is related to rumen acidosis when cattle are fed high grain diet (Golder et al., 2014). Ionophores decrease ammonia level relating to protein efficiency (McIntosh et al., 2003), and also methane production (Guan et al., 2006). These favorable changes in rumen fermentation are attributed to modulating rumen microbes: selective inhibition of hydrogen producing bacteria, mainly Gram-positive cellulolytics and some strains of methanogen (Chen and Wolin, 1979), a lactate producing

Gram-positive bacterium *Streptococcus bovis* (Russell, 1986), ammonia producing *Peptostreptococcus anaerobius* and *Clostridium sticklanii* (Krause and Russell, 1996). Ionophores also inhibit growth of protozoa and fungi (Russell and Houlihan, 2003). On the other hand Gram-negative succinate- or propionate- producing bacteria were tolerant to ionophore supplementation (Nagaraja and Taylor, 1987). This causes the enhancement of propionate level in the rumen. The mode of action of ionophores can be explained by energy shortage to expel ions accumulated in bacterial cell (Russell and Houlihan, 2003). Although ionophores have exhibited beneficial effect on rumen fermentation and animal performance, use of these additives have been gradually prohibited due to concerns on public health (McGuffey et al., 2001; Beauchemin et al., 2008). Occurrence of antibiotic resistant bacteria can be a critical threat to human. Use of avoparcin (a vancomycin analogue) also has been banned in EU (Russell and Houlihan, 2003) due to isolation of vancomycin-resistant enterococci from pigs and poultry fed avoparcin (Aarestrup, 1995; Klare et al., 1995). Thus, use of antibiotics for animals are becoming more controversial in the world.

Natural additives as an alternative to ionophores

Natural additive sources appear to be alternative to ionophores for rumen modulation. Recently, phytogenic compounds as a novel source of feed additives have been investigated, based on the fact that plants produce a large variety of metabolites showing antimicrobial activity. The use of these natural sources may not be subject to concerns raised regarding the overuse of chemically- and biologically- synthesized antibiotics including ionophores (Cowan, 1999).

Many plant-derived compounds have been reported. Salicylate, its derivatives (Lewis and

Ausubel, 2006), flavonoids and terpenoids (Greathead, 2003) have different extents of antimicrobial function. Concentration of these compounds depends on growth stage of source plant (Dudareva et al., 2004) and environmental factors such as light and temperature (Staudt and Bertin, 1998).

Attempts have been made to apply herbal sources to rumen modulation. McIntosh et al. (2003) applied blended essential oil to dairy cattle rumen in which significant inhibition of deamination of amino acid occurred. Patra and Yu (2015) monitored rumen bacterial response to origanum oil, garlic oil and peppermint oil, and found that these essential oils reduce methane production.

A methanol extract from *Sapindus rarak,* a plant rich in saponin, drastically altered SCFA composition toward less acetate and butyrate, and more propionate. The extract inhibited *R. albus*, *R. flavefaciens* and *Chytridiomycetes* (fungi) (Wina et al., 2005). However, the later long-term feeding evaluation with same material using sheep resulted in microbial adaptation to lessen the above effects (Wina et al., 2006). A recent study on saponin showed its antiprotozoal effect (Ramos-Morales et al., 2017).

It is known that plants often produce toxic substances to protect them from microbial attack (González-Lamothe et al., 2009). These substances might be toxic to animals as well (Greathead, 2003). Therefore, we have to consider this characteristic of the target plant when we use herbal sources and their compounds in animal feed.

Cashew byproduct as a new natural additive

Cashew nut shell liquid (CNSL), a byproduct of cashew nut production that accounts for about 32% of the shell, has many industrial applications and is used as a raw material for products such as paint, brake linings, lacquers and coatings (Menon et al., 1985). This liquid also exhibits a wide range of biological activities, as it contains compounds with antimicrobial (Kubo et al., 1993), antioxidative (Kubo et al., 2006) and antitumor (Itokawa et al., 1987) properties, represented by anacardic acid, cardanol and cardol, which are all salicylic acid derivatives with a carbon-15 alkyl group. These phenolic compounds, especially anacardic acid, are reported to inhibit a variety of bacteria (Kubo et al., 2003).

An early study by Van Nevel et al. (1971) first indicated that anacardic acid could be used as a propionate enhancer in the rumen. Anacardic acid is found in cashew and ginkgo trees, particularly in their seeds. The main action of anacardic acid and related phenolics is a surfactant action that inhibits mainly Gram-positive bacteria (Kubo et al., 1993) lacking an outer membrane. Such cells are physically disrupted by anacardic acid. This selective inhibition of Gram-positive rumen bacteria might result in the alteration of rumen microbiota and fermentation products.

Of alkylphenols present in CNSL, anacardic acid is most functional but decarboxylated and converted to less functional cardanol by heating and long exposure to oxygen. Watanabe et al. (2010) indicated that unheated CNSL dramatically reduced methane production while increasing propionate production in a rumen simulation technique (RUSITEC) fermenter. This was accompanied by drastic alterations in rumen microbiota that are formate and/or hydrogen producing bacteria decreased in abundance, while succinate and/or propionate producing bacteria

increased with CNSL supplementation. In feeding experiments using cattle, a similar response to CNSL was observed (Shinkai et al., 2012). However, unheated CNSL and its additive formula are not yet widely available and also waiting for further detailed evaluations.

Ginkgo byproduct and the objective of the study

Another source of anacardic acid is ginkgo plant that is grown widely among Far East Asian countries such as China, Korea and Japan. Industrial uses of ginkgo are leaves for medicine (China) and nuts for food (Korea and Japan). Leaf extracts for medicinal use are even exported to European countries. The leaf extract is also evaluated as a rumen modifier by Kim et al (2015). They found that ginkgo leaf extract decreased the abundance of protozoa and two Ruminococcal species, but the extract did not much influence rumen fermentation pattern. It is not clear if the extract contains anacardic acid, because they do not analyze alkylphenols in their preparation. Therefore, this study is not real evaluation of ginkgo leaves focusing on alkylphenols to act as a ruminant additive candidate.

Ginkgo fruit is a byproduct in the process of ginkgo nut separation (unsuitable for any uses due to its peculiar smell), yielding ca. 2,600 metric t/yr in Japan, accounted for 230% of nut production (MAFF, 2013). Therefore, biomass of ginkgo fruit is much smaller in comparison with CNSL. In this regard, use of ginkgo fruit for feed additive might be limited locally. However, no evaluation has been made so far as to the functionality of ginkgo fruit as a feed additive candidate for ruminants. Although CNSL containing anacardic acid has been evaluated and found to be an effective rumen modifier in the previous studies (Watanabe et al., 2010; Shinkai et al., 2012), its

additive formula has not yet been fully available in the world and has not been appreciated on animal feed market. It is better to have alternatives to CNSL that should be a similarly effective plant source of anacardic acid. In this regard, ginkgo fruit is a suitable alternative candidate to be evaluated in the same manner as CNSL was done.

The objective of this study was to initially screen more potent cultivar of ginkgo for the use to modulate rumen fermentation, to determine dose-response of rumen fermentation to ginkgo extract, to further evaluate microbial and fermentation changes in artificial rumen, and also to determine potency of ginkgo extract under various feeding conditions.

Chapter 2. Evaluation by batch culture, continuous culture, and pure culture studies

2.1. Introduction

Cashew nut shell liquid (CNSL) containing alkylphenols represented by anacardic acid has made favorable alterations to rumen fermentation, namely a reduction in methane and an increase in propionate production through rumen microbial selection (Watanabe et al., 2010; Shinkai et al., 2012). Anacardic acids in CNSL consist of 3 molecules with C15 alkyl side chains differing in saturation (C15:1, C15:2, and C15:3), while anacardic acids in ginkgo consist of 3 molecules possibly with different side-chain lengths according to separation pattern of alkylphenoles by HPLC (Nagashima et al, unpublished results). A previous report suggested that the length of the alkyl side chain of anacardic acid differentially affects antibacterial activity toward methicillin-resistant *Staphylococcus aureus* (Muroi et al., 2004). A variety of anacardic acids occur in a few agricultural plants that await evaluation for a wide range of applications. Of such future applications, the use of ginkgo fruit in ruminant feed is one possibility as mentioned in Chapter 1. We hypothesized that ginkgo fruit containing anacardic acid can modulate rumen fermentation without negatively affecting feed digestion. Therefore, we investigated rumen parameters, including gas, SCFA, ammonia, and microbes, to evaluate the potential of gingko fruit extract as a rumen modifier by using batch cultures, continuous cultures and pure cultures.

2.2 Materials and methods

2.2.1. Rumen fluid and donor cows

Rumen content was collected from 2 ruminally fistulated Holstein cows fed twice a day (0800 and 1700 h) with a 50% concentrate (Monster 18; Mercian, Tokyo, Japan), 50% orchardgrass hay diet at the experimental farm of Hokkaido University, Sapporo, Japan. The diet contained 18.2% CP, 41.2% NDF, and 2.16 Mcal of ME/kg on a DM basis. An equal amount of rumen content from both cows was mixed and placed in a bottle flushed with N_2 gas, then transferred to the laboratory within 30 min. The rumen content was strained through 2 layers of surgical gauze and used for in vitro experiments.

2.2.2. Ginkgo fruit and extract

We chose 2 major cultivars of ginkgo, Kyuju (K) and Tokuro (T), as candidate materials based on availability from annual production. We obtained fresh fruit after separation of the nut at a ginkgo farm in Sobue town, Aichi Prefecture, Japan, a major ginkgo-nut-producing area. Ginkgo fruit (mashed) was immediately frozen at −30°C and shipped to the laboratory. For extraction, 364 g of ginkgo fruit was suspended in 680 mL of 99.5% ethanol for 48 h, centrifuged to obtain the supernatant, and then concentrated by a centrifugal evaporator for experimental use. This stock extract was diluted with 99.5% ethanol to set each experimental dosage of ginkgo extract, expressed as percent (wt/vol) of ginkgo fruit (wet weight equivalent) in the final culture.

2.2.3. Batch culture

In vitro batch culture tests were performed as follows. Artificial saliva (McDougall, 1948) and strained rumen fluid were mixed in a 1:1 ratio (vol/vol), and 10 mL of the mixture was transferred to test tubes (180 mm length, 10 mm diameter) with a substrate that was a mixture of 0.14 g concentrate and 0.06 g orchard grass hay, identical to the mixture given to the rumen content donor cows. Ginkgo extract or an equal volume of ethanol (100 μL) was added to tubes, and the headspace was flushed with N_2 gas, sealed with a butyl rubber stopper and plastic cap, and incubated at 39°C for 24 h. For cultivar comparison, the dose of ginkgo was set at 1.6% in fruit equivalent. For dose response assays, supplementation levels of 0, 0.8, 1.6, 3.2, and 6.4% were tested. Incubation was in quintuplicate for cultivar comparison (n = 5) and quadruplicate for dose-response assays (n=4). After incubation, total gas production was measured through a needle attached pressure gauge (Aϕ60B, GL Sciences, Tokyo, Japan), and gas samples were analyzed for CO_2, CH_4, and H_2 using gas chromatography (Watanabe et al., 2010). Cultures were centrifuged, and the supernatant was used for SCFA analysis. These batch culture experiments were carried out to select the ginkgo cultivar (K vs. T) with the more potent effect and then to evaluate the dose response of rumen fermentation parameters to the selected cultivar (ginkgo K; see results).

2.2.4. Continuous culture

The rumen simulation technique (RUSITEC) (Czerkawski and Breckenridge, 1977) was used to evaluate longer-term rumen responses to ginkgo extract from the selected cultivar. The fermentation system was equipped with 8 fermentors that each had a 650 mL working capacity. The procedure for operation, including feeding and sampling, was as described by Watanabe et al.

(2010). The rumen inoculum was a mixture of strained rumen fluid from 2 cows (as used for the batch culture studies) and artificial saliva at a 5:2 ratio. Incubation lasted 7 d, consisting of 5 d for adaptation and 2 d for sampling. Artificial saliva (pH 6.8) was continuously supplied using a peristaltic pump with a dilution rate of 0.5 vol/d. The experimental diet, a ground mixture consisting of 9.1 g of concentrate and 3.9 g of orchardgrass hay, the same mixture used in the batch culture study, was fed by nylon bag to each fermentor every 24 h. Eight fermentors were divided into 2 groups (4 fermentors per group), to which ginkgo K extract in ethanol or an equal volume of ethanol (4.86 mL) was supplemented at feeding time. The dose level of ginkgo was 1.6% in fruit equivalent. Each nylon bag was taken from the fermentor after 48 h of incubation and rinsed with 20 mL of artificial saliva to recover microbes adhering to feed particles. The artificial saliva used for rinsing was returned to the fermentor at each feeding time. Rumen fluid samples were taken at 3 h intervals from each fermentor directly through a pipette. Feeding, rinsing bags, and pipette-aided sampling were done under CO_2 flushing to maintain an anaerobic atmosphere. Ruminal pH, SCFA, and ammonia were analyzed using all individual samples taken at 3 h intervals. Disappearance of DM, NDF, and ADF were determined by analyzing feed residues in nylon bag samples taken before feeding for the last 2 d. The microbial population was analyzed using culture samples before feeding for the last 2 d. Fermentation gas was collected continuously in a plastic bag (Tedlar PDF; DuPont, Wilmington, DE, USA) connected to each fermentor for the last 2 d. Total gas produced was measured by spirometer (Sanshin Kogyo, Tokyo, Japan), and gas composition was analyzed by gas chromatography (Shimadzu, Kyoto, Japan).

2.2.5. Pure culture

We cultivated representative rumen bacteria to determine the MIC of ginkgo K extract and of the purified phenolic compounds found in ginkgo: 3 anacardic acids (C13:0, C15:1, and C17:1), cardanol (C15:1), and cardol (C15:1). The rumen bacteria tested were *Ruminococcus flavefaciens* C94 (ATCC 19208) and *Ruminococcus albus* 7 (ATCC 27210) as hydrogen- and formate-producing bacteria; *Eubacterium ruminantium* GA195 (ATCC 17233), *Butyrivibrio fibrisolvens* D1 (ATCC 19171), and *Butyrivibrio proteroclasticus* (ATCC 51982) as butyrate-producing bacteria; *Lactobacillus ruminis* RF1 (ATCC 27780) and *Streptococcus bovis* (ATCC 33317) as lactate-producing bacteria; *Fibrobacter succinogenes* S85 (ATCC 19169), *Prevotella ruminicola* 23 (ATCC 19189), *Succinivibrio dextrinosolvens* 24 (ATCC 19716), *Ruminobacter amylophilus* H18 (ATCC 27944), *Succinimonas amyloytica* $B_2$4 (ATCC 19206), *Selenomonas ruminantium* GA192 (ATCC 12561); and *Megasphaera elsdenii* LC1 (ATCC 25940) as succinate- or propionate-producing bacteria or both; and *Clostridium sticklandii* (ATCC 12662), *Clostridium aminophilum* (ATCC 49906), and *Peptostreptococcus anaerobius* (ATCC 27337) as hyper-ammonia-producing bacteria. Tested concentrations of ginkgo K extract were 0, 0.05, 0.1, 0.2, 0.4, and 1.6%, and of purified phenolic compounds were 0, 1.56, 3.13, 6.25, and 12.5 µg/ mL. The extract at 1.6% was equivalent to 1.19, 6.76, and 2.67 µg/mL for anacardic acids C13:0, C15:1, and C17:1, 0.27 µg/mL for cardanol C15:1, and 1.59 µg/mL for cardol C15:1, respectively. All these pure phenolics were obtained from Idemitsu Co. Ltd. (Tokyo, Japan) and Sigma-Aldrich (Saint Louis, MO, USA). Growth of each bacterium was monitored spectrophotometrically at 660 nm. To culture all bacteria except *L. ruminis*, we used medium containing rumen fluid. The medium was prepared with 50 mL of distilled water, 7.5 mL each of mineral solutions I and II

(Bryant and Burkey, 1953), 30 mL of rumen fluid, 0.2 g of bactopeptone, 0.12 g of yeast extract, 0.1 mL of 0.1% resazurin, 0.1 g of L-cysteine HCl, 5 mL of 8% Na_2CO_3, and 0.25 g of each glucose and cellobiose. The growth medium for *L. ruminis* was MRS (Oxoid, Basingstoke, UK) containing L-cysteine HCl.

2.2.6. Chemical analysis

We analyzed the major components of the experimental feeds, ginkgo fruit, its extract, and nylon bag residue using the methods of the AOAC (1990) and VanSoest et al. (1991). We analyzed the total polyphenol and total flavonoid contents using the Folin-Ciocalteu method (Makkar et al., 1993) and the aluminum chloride method (Dewanto et al., 2002), respectively. Alkylphenolics in ginkgo fruit and ginkgo fruit extract (anacardic acids, cardanol, and cardol) were quantified by HPLC as described by Watanabe et al. (2010). Gases (H_2, CH_4, and CO_2) from microbial fermentation in batch cultures and RUSITEC were analyzed using a GC-8A gas chromatograph (Shimadzu) equipped with parallel columns of Porapak Q (Waters, Milford, MA, USA) and Molecular Sieve 13X (Restek, Bellefonte, PA, USA), and a thermal conductivity detector. We analyzed SCFA as briefly described below. Culture fluid was mixed with 25% meta-phosphoric acid at a 5:1 ratio, incubated overnight at 4°C, and centrifuged at 10,000 × g at 4°C. Then, the supernatant, mixed with crotonic acid as internal standard, was injected to a GC-14B gas chromatograph (Shimadzu) equipped with an ULBON HR-20M fused silica capillary column (0.53 mm i.d. × 30 m length, 3.0 μm film; Shinwa, Kyoto, Japan) and a flame-ionization detector. Ammonia nitrogen concentration was spectrophotometrically determined using the indophenol reaction (Weatherburn, 1967).

2.2.7. Microbial analysis

We took samples for microbiological analysis from each fermentor fluid at the time of withdrawal of the nylon bag (after rinsing but before feeding with a new nylon bag), so that both planktonic and feed-associated microbes were included. These samples were immediately frozen and kept at −80°C. Extraction of DNA for microbial analysis was performed using the repeated bead beating plus column (RBB+C) method described by Yu and Morrison (2004). The DNA was used for real-time PCR to quantify abundance of rumen representatives including total bacteria, total archaea, *R. flavefaciens, R. albus, B. fibrisolvens, Treponema bryantii, S. bovis, F. succinogenes, Prevotella bryantii, P. ruminicola, S. dextrinosolvens, R. amylophilus, Anaerovibrio lipolytica, S. ruminantium* and *M. elsdenii*. All quantitative PCR (qPCR) details such as primers, standards, PCR conditions, and calculations were as described by Koike et al. (2007) and Ohene-Adajei et al. (2008). For protozoa and fungi, details were as referred to by (Sylvester et al., 2004) and Denman and McSweeney (2006), respectively. In brief, standard plasmid containing the respective target gene sequence was obtained by PCR cloning using a target-specific primer set. The copy number of each standard plasmid was calculated using the molecular weight of nucleic acid and the length (base pair) of the cloned standard plasmid as described by Koike et al. (2007). A LightCycler system and a FirstStart DNA master SYBR I reaction kit (Roche, Penzberg, Germany) were used with 10-fold serial dilutions of standard plasmid for the respective target (16S rDNA or 18S rDNA sequence specific to each target microbe). We calculated microbial quantity using amplification curves obtained from both standard and sample. The specificity of PCR amplification was confirmed using melting curve analysis of the PCR products by increasing the temperature from 70°C to 95°C at a rate of 0.1°C/s. Microbial abundance was shown by copy

number of rDNA for total bacteria, protozoa, and fungi, or by relative proportion in total bacterial copy number for total archaea and specific bacteria.

To comprehensively analyze the microbial community, we used DNA samples from RUSITEC for MiSeq analysis (Illumina Inc., San Diego, CA, USA). Sequencing was performed by Hokkaido System Science Co. Ltd. (Sapporo, Japan). The V3 to V4 regions were amplified using 2 primer sets: S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) for bacterial rDNA (Herlemann et al., 2011), and arch349F (5′-GYGCASCAGKCGMGAAW-3′) and arch806R (5′-GGACTACVSGGGTATCTAAT-3′) for archaeal rDNA (Takai and Horikoshi, 2000). Polymerase chain reaction was carried out in a 50-μL total volume: 10 μL of 5× PrimeSTAR Buffer, 4 μL of dNTP mixture (2.5 mM each), 0.5 μL of PrimeSTAR HS DNA polymerase (Takara Bio Inc., Kusatsu, Japan), 1 μL of each primer (10 pmol/μL), 32.5 μL of distilled H_2O, and 1 μL of template DNA (10 ng/μL). The following PCR conditions were used: 30 cycles for bacteria and 40 cycles for archaea, consisting of denaturation at 98°C (10 s), annealing at 55°C (15 s), and extension at 72°C (30 s). Amplicon sequencing was carried out using MiSeq as described by Caporaso et al. (2012). Data quality control and analyses were performed using the QIIME ver. 1.8.0 pipeline (Caporaso et al., 2010). Operational taxonomic units were generated from sequences clustered at a 97% similarity threshold using the UCLUST algorithm (Edgar, 2010). Chimeric sequences were removed from the analysis using the ChimeraSlayer algorithm (http://microbiomeutil.sourceforge.net/#A_CS). Taxonomy was assigned using the Greengenes database (ver. 13.8) (McDonald et al., 2012) at a 90% similarity threshold. Difference in biodiversity between control and ginkgo extract was compared by α diversity metrics: Chao1,

Shannon index, phylogenetic diversity whole tree, observed number of operational taxonomic units, and Good's coverage.

2.2.8. Statistical analysis

The data from batch culture studies were averaged (cultivar comparison, n = 5; dose response, n = 4) for ANOVA using the GLM procedure of SPSS version 16.0 J (IBM, Tokyo, Japan). Tukey's method was employed for multiple comparison. Orthogonal contrasts were used to test linear and quadratic effects of dose level of ginkgo K extract. In the RUSITEC experiment, the data (including pH, SCFA, and ammonia) were subjected to ANOVA using the MIXED procedure of SPSS with repeated measures. The model included the effect of treatment, time after feeding, their interaction, random effects of fermentors within treatments, and residual error. Time after feeding was used as the repeated measure. Data on gas, NDF, ADF, and DM disappearance, qPCR and next-generation sequencing (MiSeq) from samples taken at 0 h (before feeding) for 2 d from each fermentor were averaged and compared (control vs. ginkgo) by Student's *t-test*. Statistical significance was considered at $P < 0.05$.

2.3. Results

2.3.1. Chemical composition of ginkgo fruit

The chemical composition of 2 cultivars of ginkgo fruit is shown in Table 1. The chemical content of the cultivars was very different: K had a higher percentage of NDF and ether extract, and lower nonstructural carbohydrate than T; K also had a higher content of total flavonoids than T. Both cultivars contained 3 types of anacardic acids, which were classified by length of the alkyl side chain and the number of double bonds (C13:0, C15:1, and C17:1). Ginkgo fruit also had cardanol (C15:1) and cardol (C15:1). The total content of these alkylphenols was more than 5 times higher in K than in T. The chemical composition of the ethanol extract of ginkgo fruit was essentially similar to that of the fruit: that is, K extract showed higher contents of ether extract, total polyphenol, total flavonoids, and alkylphenols represented by anacardic acid than T extract. Nonstructural carbohydrate content was higher in T extract than in K extract.

Table 1. Chemical composition of ginkgo fruit from cultivar Kyuju and Tokuro

Content	Kyuju	Tokuro
Dry matter, g/kg	200	284
Ingredient, g/kg DM		
Crude protein	67	60
Crude ash	38	53
Neutral detergent fiber	202	63
Non-structural carbohydrate	337	690
Ether extract	356	134
Total polyphenol	38	35
Total flavonoid	33	8
Alkylphenols	0.272	0.050
Total anacardic acid	0.231 (85.0)	0.043 (86.8)
C13:0	0.026 (9.5)	0.005 (10.3)
C15:1	0.147 (54.1)	0.027 (55.0)
C17:1	0.058 (21.4)	0.011 (21.5)
Cardanol		
C15:1	0.006 (2.2)	0.001 (2.3)
Cardol		
C15:1	0.035 (12.7)	0.005 (10.9)

Values in parenthesis are % on dry basis for major components and relative proportion of each phenolic in alkyl phenolics, repectively.
Extract was made using 0.67 g of fresh fruit/mL of ethanol.

2.3.2. Batch culture study

A comparison of the potency of the 2 ginkgo cultivars for modulating rumen fermentation is shown in Table 2. Although extracts from both cultivars greatly decreased methane production, K showed greater potential than T (86 vs. 66%). We observed hydrogen accumulation following the addition of both ginkgo extracts, but this finding was not significant. Total SCFA concentration was not changed by either ginkgo extract compared with the control. The concentration and molar proportion of acetate was decreased by K but not by T. Both ginkgo extracts enhanced propionate production. We observed lower butyrate production for K. Based on these observations, we selected K as the more potent additive for modulating rumen fermentation and used it for further study.

The dose response of fermentation to ginkgo K extract is shown in Table 3. Total gas production decreased at ≥3.2% supplementation. Methane production was linearly inhibited in a dose-dependent manner. Supplementation at >3.2% was quite effective for decreasing methane, but hydrogen accumulation occurred at ≥3.2%. Total SCFA concentration was not affected by the ginkgo extract. Acetate production linearly decreased, but propionate production linearly increased, depending on dose. The concentration and molar proportion of butyrate decreased at higher levels (≥3.2%). All parameters except CO_2 production showed a linear increase or decrease in dose response. Based on the observation of a decrease in total gas production and accumulation of hydrogen at ≥3.2%, we selected the 1.6% dose of ginkgo fruit equivalent for further evaluation.

Table 2. Comparison of two different cultivars of ginkgo (Kyuju and Tokuro) in alteration of rumen fermentation

Fermentation Parameters	Control	Extract from cultivar of*		SEM	*P*-value
		Kyuju	Tokuro		
	Gas production, mL				
Total gas	11.38[a]	10.60[b]	11.78[a]	0.17	0.003
CO_2	9.56[c]	10.25[b]	10.98[a]	0.18	<0.001
CH_4	1.81[a]	0.26[b]	0.62[b]	0.2	<0.001
H_2	0.01	0.09	0.17	0.03	0.154
	SCFA production, m*M*				
Total SCFA	87.5[ab]	86.6[b]	93.5[a]	1.18	0.022
Acetate	47.7[a]	39.8[b]	44.3[a]	0.99	0.001
Propionate	23.8[b]	29.9[a]	28.9[a]	0.75	<0.001
n-Butyrate	12.3[b]	11.0[c]	13.9[a]	0.36	<0.001
	SCFA proportion, molar %				
Acetate	55.4[a]	46.0[b]	47.4[a]	0.99	<0.001
Propionate	27.2[b]	34.5[a]	30.9[a]	0.81	<0.001
n-Butyrate	14.1[b]	12.7[c]	14.9[a]	0.26	<0.001

[a-c] Means within row with different superscripts differ ($P < 0.05$).
*Supplementation was set at 1.6% (wt/vol) of ginkgo fruit in final culture.

Table 3. Dose response of selected ginkgo (Kyuju) extract supplementation in gas and short chain fatty acid production

Fermentation Parameters	Supplementation of ginkgo extract from Kyuju					SEM	Contrast*	
	Control	0.8%	1.6%	3.2%	6.4%		Linear	Quadratic
	Gas production, mL							
Total gas	14.24[a]	14.92[a]	14.19[a]	11.83[b]	11.59[b]	0.33	<0.001	0.01
CO_2	10.67[b]	11.78[a]	11.75[a]	10.92[ab]	11.22[ab]	0.13	0.724	0.007
CH_4	3.57[a]	3.14[a]	2.43[b]	0.87[c]	0.28[d]	0.29	<0.001	0.02
H_2	0.00[c]	0.00[c]	0.00[c]	0.05[b]	0.09[a]	0.01	<0.001	0.002
	SCFA production, m*M*							
Total SCFA	119.1	119.2	117.2	111.8	107.6	1.43	0.004	0.27
Acetate	77.8[a]	73.9[ab]	66.6[b]	55.7[c]	51.5[c]	2.38	<0.001	0.544
Propionate	23.7[d]	28.2[c]	32.9[b]	40.3[a]	41.4[a]	1.58	<0.001	0.17
n-Butyrate	13.2[a]	12.7[a]	12.7[a]	9.8[b]	8.7[b]	0.42	<0.001	0.034
	SCFA proportion, molar %							
Acetate	65.3[a]	62.0[b]	56.8[c]	49.8[d]	47.8[d]	1.53	<0.001	0.691
Propionate	19.9[e]	23.6[d]	28.1[c]	36.1[b]	38.5[a]	1.61	<0.001	0.624
n-Butyrate	11.1[a]	10.7[a]	10.8[a]	08.7[b]	08.1[c]	0.28	<0.001	<0.001

[a-c] Means within a row with different superscripts significantly differ ($P<0.05$).

[*] *P*-value for the linear and quadratic effects of ginkgo extract supplementation at 0, 0.8, 1.6, 3.2 and 6.4% in ginkgo fruit equivalent in final culture.

2.3.3. Continuous culture study

Rumen fermentation profiles and feed digestibility in RUSITEC are shown in Table 4. The dosage used was 1.6%, which was confirmed as effective without adverse alteration of rumen fermentation in batch cultures. Total gas and CO_2 production was not affected, but methane production decreased by 47% when ginkgo extract was added. We observed a small amount of hydrogen accumulation. Ginkgo extract did not influence total SCFA concentration. The concentration and molar proportion of acetate were decreased by ginkgo extract, but the corresponding values of propionate were increased. We observed no change in butyrate production. Ruminal pH was slightly but significantly decreased. Ammonia concentration deceased with ginkgo extract. Disappearance of DM, NDF, and ADF were not affected by the addition of ginkgo extract.

Abundance of rumen microbe is shown in Figure 1. The absolute abundance of total bacteria expressed as log copy number per milliliter of culture increased in the presence of ginkgo extract, but that of protozoa and fungi decreased. Other bacteria, including archaea, were compared by relative abundance of total bacteria. Most of the groups quantified showed considerable changes with ginkgo extract supplementation: total archaea, *R. flavefaciens, R. albus, T. bryantii, the genus Treponema*, and *P. bryantii* decreased; genus *Prevotella, P. ruminicola, S. dextrinosolvens, R. amylophilus, A. lipolytica, S. ruminantium*, and *M. elsdenii* increased. In particular, the abundance of *M. elsdenii* was about 7 times higher with ginkgo extract addition than with control.

Rumen microbiota were comprehensively analyzed by MiSeq and compared for control and treatment (ginkgo extract). The number of sequences passing the quality filter was 102,817 for control (minimum = 67,318; median = 85,305) and 59,762 for treatment (minimum = 29,116;

median = 66,186). For archaea, 57,253 sequences were obtained from samples of control (minimum = 31,844; median = 55,318) and 51,057 for treatment (minimum = 22,631; median = 53,841). Changes in biodiversity indices inferred from bacterial and archaeal sequence reads are shown in Table 5. The high levels of Good's coverage suggest that sequences from the control and treatment represented the majority of bacteria and archaea present in the cultures. The richness and evenness of the bacterial and archaeal community decreased with treatment. The total number of operational taxonomic units, Chao1 and Shannon indices, and phylogenetic diversity (PD_Whole_tree) for both bacteria and archaea were lowered by treatment. Thus, all indices for biodiversity decreased with ginkgo treatment.

Changes in bacterial and archaeal communities at different taxonomic levels with ginkgo extract supplementation are shown in Figure 2. For bacteria at the phylum level (Figure 2A), the abundance of *Bacteroidetes* decreased with ginkgo extract (43.3 vs. 24.1%), but the abundance of *Firmicutes* increased (35.3 vs. 44.0%). The abundance of *Proteobacteria* was 4.6 times higher with ginkgo extract (3.9 vs. 18.0%). More drastic change was seen for *Synergistes*, showing a 41-fold increase with ginkgo extract (0.1 vs. 4.6%), of which *Pyramidobacter* was the main member. At the genus level (Figure 2B), increased abundance with ginkgo extract was seen for specific groups related to succinate and propionate production, or both. These were *Succinivibrio* (0.6 vs. 9.8%), *Ruminobacter* (2.2 vs. 7.2%), *Selenomonas* (0.5 vs. 4.1%), *Megasphaera* (6.7 vs. 22.6%), and *Prevotella* (14.5 vs. 19.4%). At the same time, decreased abundance was seen for *Fibrobacter* (2.5 vs. 0.7%) and unclassified bacteria belonging to the *Bacteroidales* (19.7 vs. 1.6%), *Clostridiales* (4.4 vs. 0.4%), and *Ruminococcaceae* (6.6 vs. 0.4%). The archaea community in the control comprised 3 classes: *Methanobacteria* (40.1%), *Methanomicrobia* (25.6%), and *Thermoplasmata*

(19.7%). By adding ginkgo extract, the community was changed toward more *Methanomicrobia* (61.4%) and less *Methanobacteria* (17.2%) and *Thermoplasmata* (0.6%) (Figure 2C). The majority of each class were the genera *Methanobrevibacter* (39.3 vs. 16.9%), *Methanoplanus* (22.3 vs. 59.8%), and vadin CA11 (*Methanomassiliicoccaceae*; 19.7 vs. 0.6%; Figure 2D).

Table 4. Effect of ginkgo (Kyuju) extract supplementation on rumen fermentation profile in RUSITEC

Parameters	Control	Ginkgo (Kyuju) extract*	SEM	*P*- value
pH	6.67	6.62	0.007	<0.001
Ammonia (mg of N/100 mL)	17.5	8.1	0.85	<0.001
	Gas production, mL/d			
Total gas	1625	1580	80.86	0.797
CO_2	1387	1442.1	73.87	0.729
CH_4	233	109.2	16.55	<0.001
H_2	5	28.9	3.87	0.003
	SCFA production, m*M*			
Total VFA	64.9	67.5	0.89	0.143
Acetate	30.1	23	0.45	<0.001
Propionate	11.3	17.3	0.34	<0.001
n-Butyrate	15.8	15.9	0.24	0.625
	SCFA proportion, molar %			
Acetate	49.2	37.9	0.71	<0.001
Propionate	18.5	28.5	0.56	<0.001
n-Butyrate	25.8	26.3	0.39	0.295
	Digestibility of, %			
DM	59.6	60.9	0.47	0.184
NDF	46.7	46.3	0.47	0.718
ADF	27.9	28.5	0.45	0.546

* Ginkgo extract was supplemented at 1.6% of ginkgo fruit in final culture.

Table 5. Comparison of bacterial and archaeal biodiversity by MiSeq analysis of RUSITEC culture

Item	OTUs	Chao1	Shannon (H´)	PD_whole_tree	Good's coverage (%)
	Bacterial community				
Control	4706	9730	8.3	261	97.2
Ginkgo (Kyuju) extract*	1728	3505	5.5	118	98.2
SEM	587	1236	0.5	28	0.2
P- value	0.007	0.01	<0.001	0.003	0.021
	Archaeal community				
Control	1398	2910	4.5	51	98.6
Ginkgo (Kyuju) extract*	761	1423	2.8	34	99.2
SEM	141	310	0.3	4	0.1
P- value	0.024	0.013	<0.001	0.019	0.036

* Ginkgo extract was supplemented at 1.6% of ginkgo fruit in final culture.

OTU: Operational taxonomic units.

Read length will be mentioned in result.

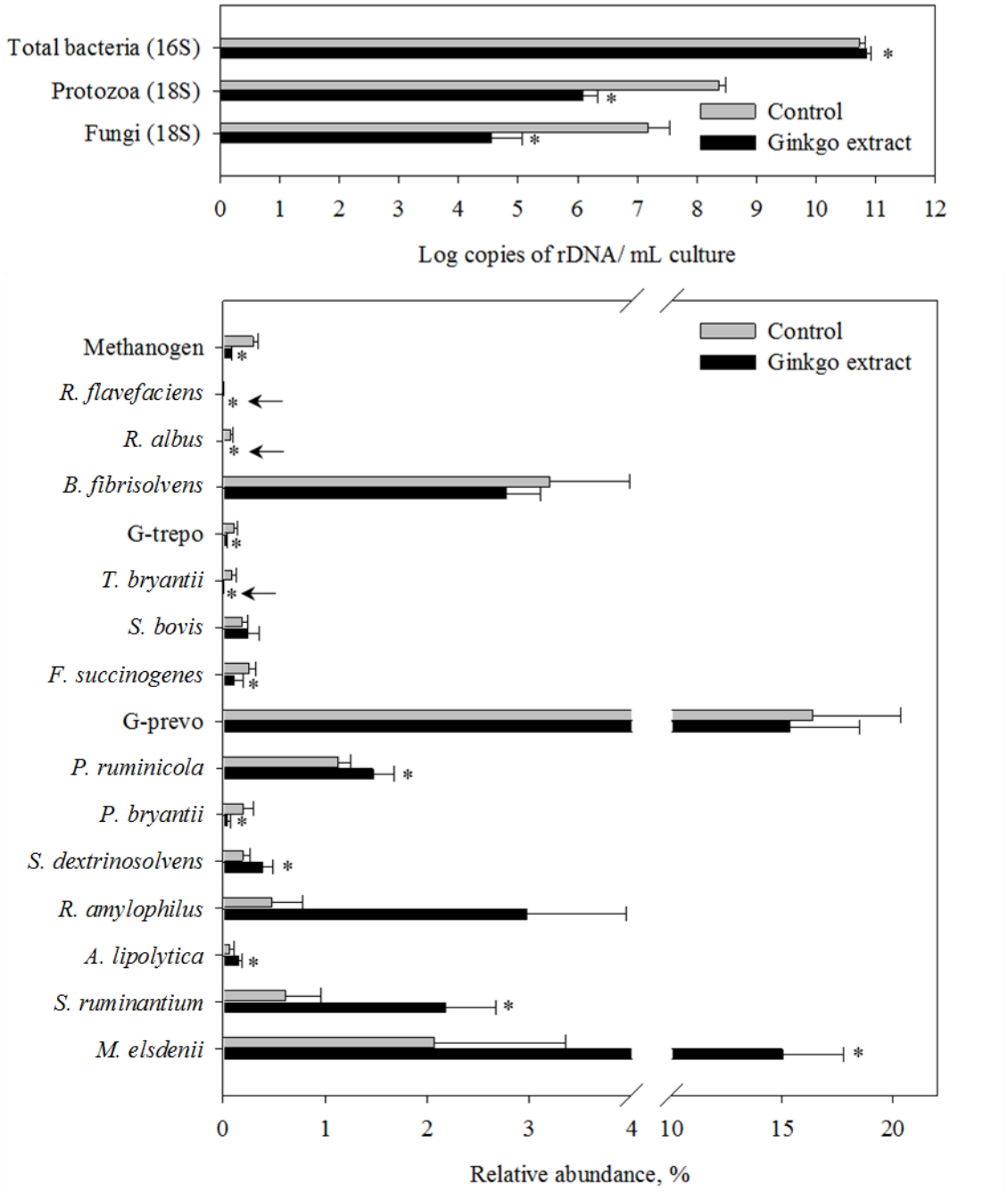

Figure 1. Relative abundance of rumen representative bacterial species and groups in RUSITEC (rumen simulation technique). Ginkgo extract was supplemented at 1.6% in fruit equivalent. G-trepo = genus *Treponema*, G-prevo = genus *Prevotella*; species include *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Butyrivibrio fibrisolvens*, *Treponema bryantii*, *Streptococcus bovis*,

Fibrobacter succinogenes, *Prevotella ruminicola*, *Prevotella bryantii*, *Succinivibrio dextrinosolvens*, *Ruminobacter amylophilus*, *Anaerovibrio lipolytica*, *Selenomonas ruminantium*, and *Megasphaera elsdenii.* Arrow indicates decrease. Asterisk (*) indicates statistical significance ($P < 0.05$). Error bar indicates standard deviation.

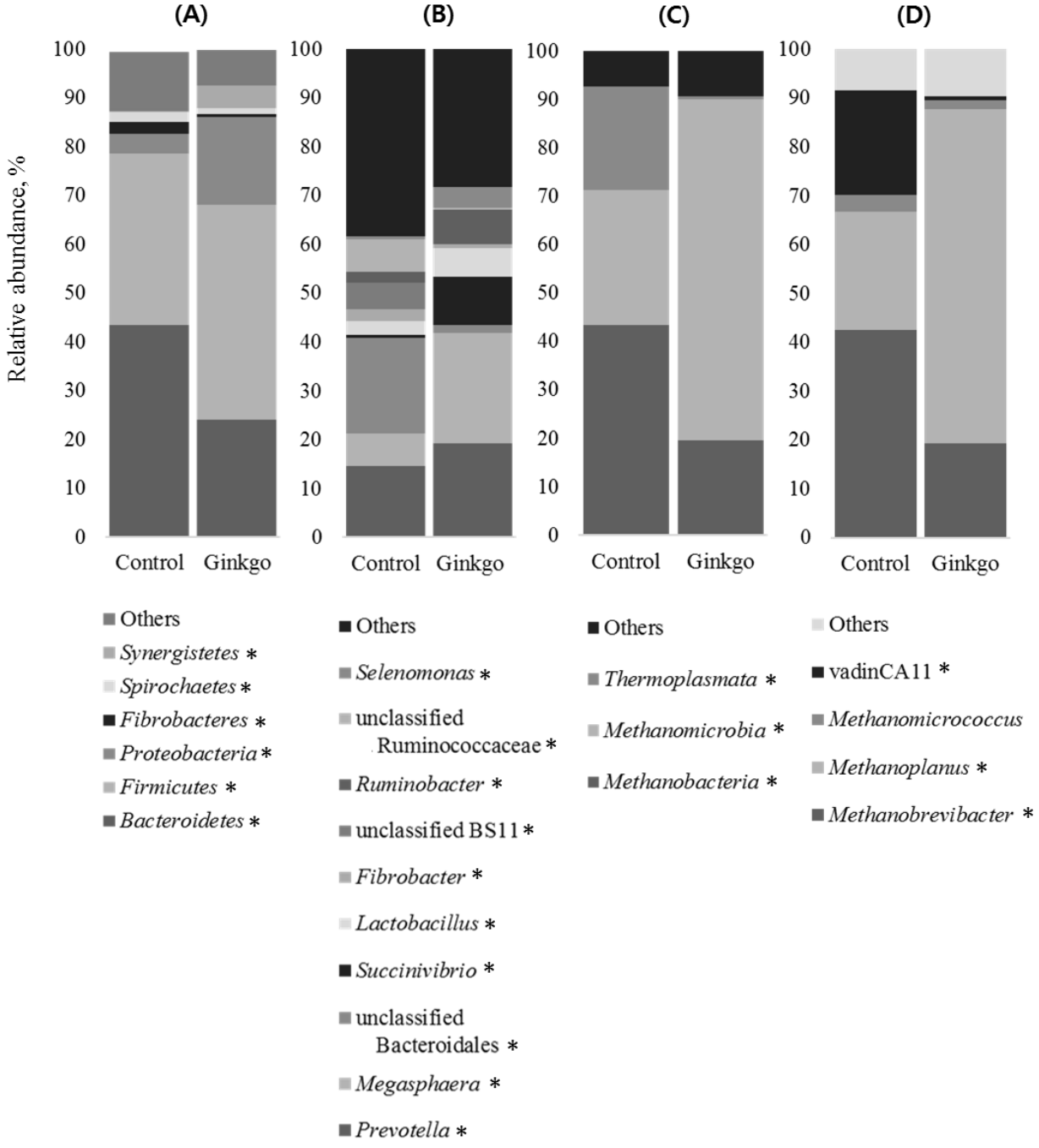

Figure 2. Effect of ginkgo extract supplementation on rumen microbiota in RUSITEC (rumen simulation technique) evaluated by MiSeq (Illumina Inc., San Diego, CA) at bacterial phylum level (A), at bacterial genus level (B), at archaeal class level (C), and at archaeal genus level (D). Asterisk (*) indicates statistical significance between control and treatment ($P < 0.05$).

2.3.4. Pure culture study

The MIC values of ginkgo extract and its phenolic composition for rumen bacteria are shown in Table 6. The bacterial response to ginkgo extract was clearly divided into sensitive (MIC ≤ 0.1%) and insensitive (MIC > 1.6%) groups. The sensitive group included *R. flavefaciens, R. albus, E. ruminantium, B. fibrisolvens, B. proteoclasticus, S. bovis, F. succinogenes, P. ruminicola, S. amyloytica, C. sticklandii, C. aminophilum,* and *P. anaerobius*. Insensitive species were *L. ruminis, S. dextrinosolvens, R. amylophilus, S. ruminantium,* and *M. elsdenii*. Hydrogen/formate producers and hyper ammonia-producing bacteria belonged to the sensitive group, but succinate- and propionate-producing (or both) bacteria were in the insensitive group. Purified cardanol and cardol, phenolic compounds present in ginkgo, did not show strong antibacterial activity to rumen bacteria except *C. sticklandii*, but all 3 anacardic acids showed selective inhibition of bacterial growth, although the anacardic acid with a C17:1 alkyl side chain was slightly less potent than the others (C13:0 and C15:1). Of the hyper ammonia-producing bacteria, *C. sticklandii* and *P. anaerobius* were sensitive to ginkgo extract and the purified phenolic compounds, but *C. aminophilum* was less sensitive

Table 6. Fermentation products of rumen representative bacteria and their sensitivity to ginkgo extract (Kyuju) and individual alkylphenolic components

Bacterial species	Fermentation products	Ginkgo (Kyuju) extract, %*	Alkylphenolic components, μg/mL				
			Anacardic acids			Cardanol	Cardol
			C13:0	C15:1	C17:1	C15:1	C15:1
Gram-positive bacteria							
Ruminococcus flavefaciens	F, A, S, H_2	<0.05	3.13	3.13	6.25	>12.5	12.5
Ruminococcus albus	F, A, E, H_2	<0.05	3.13	3.13	3.13	12.5	12.5
Eubacterium ruminantium	F, A, B, L	<0.05	6.25	3.13	6.25	>12.5	>12.5
Butyrivibrio fibrisolvens	F, A, B, L, H_2	<0.05	6.25	3.13	6.25	12.5	12.5
Butyrivibrio proteoclasticus	F, A, B, H_2	<0.05	6.25	6.25	6.25	>12.5	12.5
Lactobacillus ruminis	L	>1.6	>12.5	>12.5	>12.5	>12.5	>12.5
Streptococcus bovis	F, A, L, E	0.1	12.5	>12.5	12.5	>12.5	>12.5
Clostridium sticklandii	A, P, Br, B	<0.05	6.25	6.25	12.5	6.25	3.13
Clostridium aminophilum	A, B	0.1	12.5	>12.5	>12.5	>12.5	>12.5
Peptostreptococcus anaerobius	A, Br	<0.05	1.56	6.25	3.13	12.5	6.25
Gram-negative bacteria							
Fibrobacter succinogenes	F, A, S	<0.05	12.5	12.5	>12.5	>12.5	12.5
Prevotella ruminicola	F, A, P, S	<0.05	12.5	12.5	12.5	>12.5	>12.5
Succinivibrio dextrinosolvens	F, A, S, L	>1.6	>12.5	>12.5	>12.5	>12.5	>12.5
Ruminobacter amylophilus	F, A, S	>1.6	>12.5	>12.5	>12.5	>12.5	>12.5
Succinimonas amyloytica	A, P, S, H_2	<0.05	6.25	3.13	>12.5	12.5	12.5
Selenomonas ruminantium	A, P, B, L. H_2	>1.6	>12.5	>12.5	>12.5	>12.5	>12.5
Megasphaera elsdenii	A, P, Br, B, H_2	>1.6	>12.5	>12.5	>12.5	>12.5	>12.5

* Percentage of ginkgo fruit in the final culture.

A, acetate; B, butyrate; Br, branched chain fatty acid; E, ethanol; F, formate; H_2, hydrogen (gas); L, lactate; P, propionate; S, succinate

2.4. Discussion

2.4.1. Alkylphenols and other compounds of ginkgo fruit

Of the alkylphenols, anacardic acid was most abundantly present in ginkgo fruit and its extract (Table 1). Although the alkyl side-chain structure is different, the anacardic acids in ginkgo were potent for inhibiting specific rumen bacteria (Table 6), as found for the anacardic acids in CNSL (Watanabe et al., 2010). This suggests that ginkgo fruit is as potent as CNSL in altering the rumen microbial community via the surfactant action of alkylphenols (Kubo et al., 1993) to lead fermentation changes, if dosed at a suitable level (Watanabe et al., 2010; Shinkai et al., 2012).

Ginkgo fruit contains a greater proportion of anacardic acids than other parts (leaves or seeds) that have been commercially used (van Beek and Montoro, 2009). Therefore, the fruit is more functional as an antimicrobial and more easily available in terms of quantity than leaves and seeds. As predicted, we observed a difference in functionality between ginkgo cultivars (Table 2), and we identified K, widely distributed as the main cultivar, as a better feed additive candidate for ruminant animals. Compared with ginkgo K, ginkgo T had a lower alkylphenol content and a higher nonstructural carbohydrate content, including polysaccharides that might interact with alkylphenols, reducing the potency of such phenols, or more simply, making phenols physically less available for microbiota (Cieslak et al., 2014). This could be why T was less potent than K. Based on total flavonoid and nonstructural carbohydrate contents, other bioactive compounds besides alkylphenols in ginkgo extract might influence rumen fermentation; for example, flavonoid-rich plant extracts decrease rumen methane (Kim et al., 2015), and ginkgo leaf polysaccharides possess antioxidant activity (Jiao et al., 2016). More details on the mode of action of ginkgo extract is needed, but alkylphenols could be a main factor in altering rumen microbiota

and fermentation (discussed later). In application, one problem needs to be solved: ginkgo fruit contains the nutritionally harmful 4-methylpyridoxin (a vitamin B_6 antagonist) (Isah, 2015), which may be unsuitable for animal feed. If the activity of this antinutritional compound can be reduced by biological treatments such as silage fermentation, then ginkgo fruit can be further evaluated in animal feed studies. Some lactic acid bacteria can produce vitamin B_6 (Champagne et al., 2010), which might compensate for the negative effects of 4-methylpyridoxin if those bacteria are used as a silage inoculant. This possibility needs to be confirmed to better assess the functionality of ginkgo fruit as a potential feed additive for ruminant animals.

2.4.2. Rumen fermentation and digestion

Based on the RUSITEC study results, supplementation with ginkgo fruit extract altered the rumen fermentation pattern with no adverse effect on feed digestion (Table 4). We observed a significant decrease in methane production without affecting total gas production. Although the SCFA pattern was greatly changed by ginkgo extract toward less acetate and more propionate production, total SCFA levels were not changed. At the same time, and most importantly, the disappearance of DM, NDF, and ADF were not affected by ginkgo extract. This is the most addressed characteristic for developing a new additive candidate, as reported for CNSL (Watanabe et al., 2010). Indeed, such great shifts in rumen fermentation products are suggestive of the occurrence of rumen microbial changes (see below). The selective inhibition of specific microbes by these additives should be compensated for by activation of alternative microbes to maintain rumen fermentation within normal conditions. Hydrogen accumulation with ginkgo supplementation was observed but was minimal, suggesting the quick development of an electron

sink pathway alternative to methanogenesis, such as propionate production (Kobayashi, 2010). However, it is important to optimize the supplementation level of ginkgo extract, because an overdose (>3.2% as fruit equivalent) can depress rumen fermentation, as shown in our dose-response experiments, where total gas production was inhibited (Table 3).

The rumen ammonia level was markedly reduced (53.7%) by supplementation with ginkgo extract in RUSITEC (Table 4). In a similar RUSITEC study (Watanabe et al., 2010), CNSL decreased rumen ammonia level by 16.5%. The decreased rumen ammonia level might lead to the improvement of feed N economy if the ammonia level is higher than the critical level for maintaining microbial protein synthesis (5 mg of N/dL) (Griswold et al., 2003). The ammonia N level in the ginkgo-supplemented fermentor was 8.1 mg of N/ dL, which could meet this requirement. The observed decreased ammonia level could be related to the abundance of hyper-ammonia-producing bacteria, including *P. anaerobius*, *C. sticklandii*, and *C. aminophilum* (Paster et al., 1993). In fact, they were quite sensitive to ginkgo extract and its component anacardic acids (Table 6). Protozoa contribute to ammonia production in the rumen (Patra and Yu, 2014). Therefore, ammonia reduction observed in RUSITEC supplemented with ginkgo extract could be partly related to the decrease in protozoal population size (Figure 1). Overall, although rumen responses to ginkgo extract were quite apparent, these observations were based on short-term incubation (up to 7 d in RUSITEC). Longer incubation or feeding studies are needed.

2.4.3. Microbial community

Rumen fermentation changes due to ginkgo extract were reasonably attributable to microbial community changes evaluated by qPCR (Figure 1) and MiSeq (Figure 2) analyses, and by sensitivity of individual rumen bacteria to the extract (Table 6). Microbial selection by ginkgo extract was suggested by growth inhibition of hydrogen- and formate-producing bacteria (*R. flavefaciens* and *R. albus*) and by growth stimulation of succinate- or propionate-producing bacteria (*P. ruminicola*, *S. dextrinosolvens*, *R. amylophilus*, *S. ruminantium*, and *M. elsdenii*) according to qPCR and MIC results. The MiSeq results confirmed this. Although butyrate producers (*E. ruminantium*, *B. fibrisolvens*, and *B. proteoclasticus*) were quite sensitive to ginkgo extract, as seen by the MIC, butyrate production in batch cultures or RUSITEC was not decreased. This was likely due to the increased abundance of *M. elsdenii*, which produce butyrate as another metabolite. The qPCR-observed decrease in number of protozoa and fungi, which are hydrogen and formate producers, respectively, is also indicative of these fermentation changes. Although the abundance of fibrolytic organisms (*Fibrobacter*, *Ruminococcus*, fungi) was depressed by ginkgo extract supplementation, NDF and ADF digestibility was not affected. This suggests development of an alternative fibrolytic system, possibly due to an increase in abundance of *Prevotella* and *Selenomonas* (Figure 2), some groups of which are involved in fiber degradation (Bekele et al., 2010; Sawanon et al., 2011). The contribution of uncharacterized fibrolytic bacteria (Kobayashi, 2006; Jami et al., 2012) may also need to be considered. Although reduced protozoa and fungi may cause the decrease of fiber degradation, protozoa maintained in RUSITEC were non-fibrolytic entodinia (Béra-Maillet et al., 2005), and fungi might have already been selected for less fibrolytic ones in the 70% concentrate diet used in this study. Because the microbial situation influenced by

ginkgo may differ between diets, evaluation of a high-fiber diet needs to be conducted to draw more general conclusions.

The decrease in methane with ginkgo extract supplementation in RUSITEC could be explained not only by a shift in the hydrogen-utilizing pathway to facilitate fumarate reduction and the acrylate pathway (both responsible for propionate production) but also by partial inhibition of methanogens. Decreased abundance of total methanogens, protozoa, and fungi by ginkgo (Figure 1) indicates both direct and indirect inhibition of methanogenesis, because planktonic methanogens and eukaryote (protozoa and fungi)–associated methanogens (Vogels et al., 1980; Cheng et al., 2009) are involved in methane synthesis. From MiSeq analysis for rumen archaea, 2 dominant genera were identified and showed different responses to ginkgo extract in their relative abundance: a decrease in abundance of *Methanobrevibacter* and an increase in *Methanoplanus*. Our data (unpublished results) suggest that sensitivity of methanogens to anacardic acids depends on the genus and even the species within the same genus. Because anacardic acids act as surfactants, differences in the cell surface structure of rumen methanogens might result in differential sensitivity. The *Methanoplanus petrolearius* type strain has glycoprotein in its cell envelope (Brambilla et al., 2010), which may confer tolerance to surfactants such as anacardic acids. This indicates the occurrence of archaeal selection. However, the contribution of *Methanoplanus* to methane production is still unclear. Another interesting change due to supplementation with ginkgo extract was the decrease in number of *Methanomassiliicoccaceae*. Attention is being paid to this group of methanogens because of their contribution to methane production based on meta-transcriptomic analysis of cattle rumen (Poulsen et al., 2013); methylcoenzyme M reductase transcripts from this group decrease with dietary supplementation of rapeseed oil to decrease

methane production. Ivy fruit saponin, another rumen modifier candidate, also reportedly decreases the abundance of this group of methanogens with a concomitant decrease of methane in RUSITEC (Belanche et al., 2016). The *Methanomassiliicoccaceae* may be a good target for mitigating rumen methane in the future strategy.

2.5. Conclusion

We evaluated the potential of ginkgo fruit as a feed additive in a series of in vitro experiments. Ginkgo extract obtained from the Kyuju cultivar significantly decreased ruminal methane and ammonia, and it enhanced propionate production. These effects were caused by selective inhibition of specific rumen microbes via surfactant action of the alkylphenols present in ginkgo fruit. Therefore, ginkgo fruit is a potential candidate as a feed additive for ruminant animals.

Chapter 3. Evaluation by batch culture with different dietary conditions

3.1. Introduction

Methane emitted from ruminant animals is recognized as an undesired product based on our knowledge of the greenhouse effect and dietary energy loss (Johnson and Johnson, 1995). In order to mitigate methane production in the rumen, several strategies such as dietary treatment (Beauchemin et al., 2008), ionophores (Guan et al., 2006), essential oils (Patra and Yu, 2012), vaccination (Wedlock et al., 2013) and defaunation (Morgavi et al., 2012) have been investigated at various scales. Recently, phytogenic compounds as novel sources of feed additives have been investigated based on the fact that plants produce a large variety of metabolites, and the use of these natural sources may not be subject to concerns raised regarding the overuse of chemically synthesized antibiotics (Cowan, 1999). In chapter 2, ginkgo fruit, which contains antimicrobial phenols such as anacardic acid, was described for its potency to modulate rumen fermentation by using *in vitro* culture evaluation systems. The functionality of this candidate additive was assessed in a fixed single dietary regimen and ginkgo fruit extract was found to select for rumen microbes, thereby altering fermentation towards more propionate and less methane production.

These strategies could alter rumen modulation to various extents, as the rumen microbiota is affected by feed carbohydrate type and the resultant changes in pH (Hook et al., 2010; Veneman et al., 2015). For instance, ionophore effects on metabolic energy level vary depending on the dietary conditions and the associated ruminal responses (Guan et al., 2006). Therefore, to enable wider application of ginkgo fruit as a novel candidate additive in the future, the potential of this candidate additive must be evaluated under various feeding regimens. This chapter describes about an *in vitro* batch culture study to assess ruminal responses to ginkgo fruit

extract supplementation of the dietary substrate with different forage to concentrate ratios.

3.2. Materials and methods

3.2.1. Rumen fluid and donor cows

Two Holstein dry cows fitted with rumen cannula were employed for sampling of rumen contents at the experimental farm of Hokkaido University, Sapporo, Japan. Animals were fed twice daily (08:00 and 17:00 hours) with a 50% concentrate (Monster 18; Mercian, Tokyo, Japan) and 50% orchard grass hay diet, which contained 13.8% crude protein (CP), 54.4% neutral detergent fiber (NDF), and 2.16 Mcal metabolizable energy (ME)/kg on a dry matter basis. Samples of rumen contents were obtained from the cows, mixed in equal proportions, placed in a bottle, flushed with N_2 gas and transferred to the laboratory within 30 min. Then, the rumen content was strained through 2-layers of surgical gauze for use in the in vitro experiments. All the study protocols conformed to the Guidelines for Animal Experiments, Hokkaido University (2007) and the Act on the Welfare and Management of Animals (2005).

3.2.2. Ginkgo fruit extract

The ginkgo cultivar Kyuju was selected as the source material, as it was identified to be a more potent modulator of rumen fermentation than Tokuro, another main cultivar (Oh et al., 2017). The ginkgo fruit was physically mashed and separated using a hand-made machine, and sampled at a private ginkgo farm in Sobue, Aichi Prefecture, Japan, which is a major ginkgo nut producing area. The ginkgo fruit samples were frozen at -30°C prior to shipping to the laboratory. To prepare the ginkgo extract, 20 g of thawed ginkgo fruit was soaked in 50 mL of 99.5% ethanol for 48 hours,

subjected to centrifugation (10,000×g, 10 min), and the resultant supernatant was used as the ginkgo extract. The supernatant was concentrated using a centrifugal evaporator (VC-15SP; TAITEC, Koshigaya, Japan) and adjusted to the final concentration (1.6 g/mL ethanol, fruit equivalent) prior to use in experiments.

3.2.3. Batch culture

An *in vitro* batch culture experiment was carried out to compare the effect of ginkgo extract supplementation on rumen modulation under five different forage to concentrate (F:C) ratios. Equal volumes of strained rumen fluid and artificial saliva (McDougall, 1948) were mixed, then 10 mL of this mixture was transferred into a test tube (180 mm length, 10 mm diameter) with 0.2 g of feed mixture consisting of orchard grass hay and concentrate (both ground and passed through a 1 mm sieve) at five F:C ratios (1:9, 3:7, 5:5, 7:3 and 9:1). The hay and concentrate were the same as fed to the rumen fluid donor cows. An equal volume (0.1 mL) of ethanol (99.5%) or ginkgo extract (1.6% fruit equivalent in final concentration) was added to the test tube for control and treatment, respectively. The test tube was flushed with N_2 gas, sealed with a butyl rubber stopper and plastic screw cap, and incubated at 39°C for 24 h. Incubation was performed for 5 replicates for each treatment and dietary substrate. After incubation, total gas production was measured through a needle-attached pressure gauge (Aϕ60B; GL Science, Tokyo, Japan), and individual gases (CO_2, CH_4 and H_2) were analyzed by gas chromatography as described by (Watanabe et al., 2010). Cultures were sampled and then stored at -30°C for short chain fatty acid (SCFA) and ammonia nitrogen analyses, and -80°C for microbial analysis.

3.2.4. Chemical analysis

Chemical analysis of phenolic compounds, fermentation gases and SCFAs was carried out as described in Chapter 2. The presence of anacardic acid, cardanol and cardol in the ginkgo extract was determined by HPLC (Watanabe et al., 2010). Fermentation gases were analyzed using a gas chromatography (GC-8A; Shimadzu, Kyoto, Japan) installed with parallel columns, Porapak Q (Waters, Milford, MA, USA) and Molecular Sieve 13X (Restek, Bellefonte, PA, USA), and a thermal conductivity detector. SCFA sample preparation and gas chromatographic determination were the same as described in Chapter 2. In brief, SCFA was separated and detected using a gas chromatograph (GC-14B; Shimadzu, Kyoto, Japan) with a fused silica capillary column (ULBON HR-20M, 0.53 mm i.d. × 30 m length, 3.0 μm film; Shinwa, Kyoto, Japan) and a flame ionization detector. Ammonia nitrogen in the culture was measured by the indophenol reaction (Weatherburn, 1967).

3.2.5. Microbial analysis

Real-time PCR assay was employed to determine the microbial composition in response to ginkgo supplementation of different substrates. DNA was extracted from the culture fluids of various F:C ratios (1:9, 5:5 and 9:1) using the repeated bead beating plus column method (Yu and Morrison, 2004) and applied to real-time PCR reaction specific to the following target species: total bacteria, total methanogens, Butyrivibrio group, *Ruminococcus flavefaciens, Ruminococcus albus, Treponema bryantii, Streptococcus bovis, Fibrobacter succinogenes, Prevotella bryatii, Prevotella ruminicola, Succinivibrio dextrinosolvens, Ruminobacter amylophilus, Anaerovibrio lipolytica, Selenomonas ruminantium* and *Megasphaera elsdenii.* The quantitative PCR

procedures including primers, standards, temperature conditions and calculations were the same as previously described (Ouwerkerk et al., 2002; Koike et al., 2007; Boeckaert et al., 2008; Ohene-Adajei et al., 2008). In brief, plasmid (10-fold serial dilution) containing the 16S rDNA sequence specific to the target species was used as a standard. Amplification was carried out using a Lightcycler Firststart DNA master SYBR I reaction kit (Roche, Penzberg, Germany), and PCR products were subjected to melting curve analysis (70°C to 95°C at a rate of 0.1°C/s) to confirm the specificity of PCR amplification. Bacterial levels were calculated based on amplification curves derived from the sample and standard, and expressed as copy number of 16S rDNA for total bacteria and as relative % of total bacteria for specific species/groups.

3.2.6. Statistical analysis

The data on rumen fermentation (n = 5) with ginkgo extract supplementation under different F:C ratios were subjected to ANOVA using the general linear model procedure of SPSS (Version 16.0 J, Tokyo, Japan) as follows: $Yij = \mu + \tau i + \beta j + \gamma ij + \epsilon ijk$, where Yij is the observation, μ is the overall mean, τi is the effect of treatment, βj is the effect of diet, γij is interaction between treatment and diet, and ϵijk is residual error. Tukey's method was used for multiple comparisons across 5 and 3 different F:C ratios (for fermentation products and microbes, respectively) and 2 treatments (control and ginkgo extract).

3.3. Results

Chemical components of tested substrates (hay and concentrate) and ginkgo extract as a supplement are shown in Table 7. Ginkgo cultivar used in this experiment was Kyuju evaluated in

Chapter 2, but its fruit was collected in different year. Therefore, contents and proportions of each alkylphenols were slightly different between extracts tested in Chapter 2 and this experiment. However, the major alkylphenol in both extracts was anacardic acid, especially anacardic acid C15:1.

In vitro fermentation gas and SCFA profiles are shown Table 8. Total gas production for each dietary substrate was not affected by ginkgo extract supplementation, except for the observed decrease with the 5:5 ratio diet. Methane decreased with ginkgo extract supplementation, with the greatest reduction found with the 5:5 ratio (41.9%); whereas, the F:C ratio itself had no marked effect on methane production. Hydrogen was accumulated following ginkgo extract supplementation but not following ethanol treatment (control). Hydrogen accumulation was observed at 3:7, 5:5, 7:3 and 9:1 F:C ratios. Total SCFA concentration was not affected by ginkgo extract supplementation in each dietary substrate. However, total SCFA was clearly affected by the F:C ratio, and decreased as the proportion of concentrate in the substrate decreased. Concentrations and proportions of acetate and propionate were affected by both ginkgo extract supplementation and diet; a decrease of acetate and increase of propionate were observed for the 5 F:C ratios tested. A higher F:C ratio resulted in higher acetate and lower propionate proportions. Ammonia did not show a consistent alteration with ginkgo extract supplementation and the different F:C ratios.

The effect of ginkgo extract supplementation on bacterial levels is shown in Tables 9. Total bacteria was decreased by ginkgo extract supplementation. The relative level of fibrolytic bacteria such as *R. flavefaciens, R. albus and F. succinogenes* was decreased by ginkgo extract supplementation, and the decrease was dependent on both the species and F:C ratio; the predominant *R. albus* showed obvious decreases at 5:5 and 9:1 ratios. Several species related to

succinate and propionate production, such as *S. ruminantium, A. lipolytica, R. amylophilus, S. dextrinosolvens* and *M. elsdenii,* were increased with ginkgo extract supplementation under almost all dietary substrate conditions, except for *R. amylophilus* and *S. dextrinosolvens,* which did not show a clear response to the high concentrate diet (1:9 ratio).

Table 7. Chemincal composition of experimental substrate and supplemented ginkgo fruit extract.

Content	Hay	Concentrate	Ginkgo extract*
DM, g/kg	971	969	88
Ingredient, g/kg DM			
Crude protein	74	203	2
Crude ash	63	61	3
Ether extract	9	97	424
Neutral detergent fiber	743	345	-
Acid detergent fiber	448	134	-
Non-structural carbohydrate	112	294	571
Total polyphenol	-	-	71
Total flavonoid	-	-	62
Alkylphenolics	-	-	9.2
Anacardic acid (C13:0)	-	-	1.1
Anacardic acid (C15:1)	-	-	4.9
Anacardic acid (C17:1)	-	-	1.9
Cardanol (C15:1)	-	-	0.2
Cardol (C15:1)	-	-	1.2

*Extraction was made using 1.6 g fresh fruit/mL absolute ethanol (99.5%).

Table 8. Effect of ginkgo extract supplementation under different forage to concentrate ratios on gas, SCFA and ammonia production

Fermentation parameters	Forage to concentrate ratios										SEM	*P*-value		
	1:9		3:7		5:5		7:3		9:1					
	Control*	Treatment*	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment		Additive	Diet	A×D
Gas production, mL														
Total gas	13.4^{a}	13.2^{ab}	12.3^{abcd}	12.6^{abc}	13.1^{ab}	11.8^{cde}	12.1^{bcd}	11.3^{de}	11.1^{de}	10.6^{e}	0.15	0.005	<0.001	0.062
CO_2	9.5^{ab}	9.8^{a}	9.0^{bc}	9.8^{a}	8.8^{cd}	9.3^{b}	8.2^{e}	8.8^{cd}	7.9^{e}	8.3^{de}	0.09	<0.001	<0.001	0.363
CH_4	4.0^{a} (100)	3.4^{ab} (85.4)	3.4^{ab} (100)	2.8^{b} (84.0)	4.3^{a} (100)	2.5^{b} (58.1)	4.0^{a} (100)	2.5^{b} (63.3)	3.2^{ab} (100)	2.3^{b} (71.7)	0.11	<0.001	0.006	0.037
H_2	nil	nil	nil	0.003	nil	0.006	nil	0.006	nil	0.007	0.001	0.000	0.268	0.268
SCFA production, m*M*														
Total SCFA	12.8^{ab}	13.0^{a}	12.3^{bc}	12.7^{abc}	12.2^{cd}	11.8^{de}	11.6^{ef}	11.0^{fg}	11.0^{g}	10.6^{g}	0.12	0.044	<0.001	0.001
Acetate	8.1^{a}	7.7^{b}	8.1^{a}	7. 6^{b}	8.1^{a}	7.0^{c}	7.9^{ab}	6.8^{c}	7.6^{b}	6.8^{c}	0.07	<0.001	<0.001	<0.001
Propionate	2.6^{bc}	3.3^{a}	2.4^{cd}	3.3^{a}	2.3^{de}	3.1^{a}	2.1^{ef}	2.7^{cd}	2.0^{f}	2.5^{b}	0.07	<0.001	<0.001	<0.001
iso-Butyrate	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.00	0.466	0.540	0.484
n-Butyrate	1.7^{a}	1.6^{a}	1.5^{b}	1.4^{b}	1.4^{b}	1.2^{c}	1.2^{c}	1.2^{c}	1.0^{d}	1.0^{d}	0.03	0.005	<0.001	<0.001
iso-Valerate	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.2	0.2	0.2	0.00	0.005	0.094	0.431
n-Valerate	0.1^{a}	0.1^{abc}	0.1^{ab}	0.1^{bc}	0.1^{bc}	0.1^{d}	0.1^{c}	0.1^{c}	0.1^{d}	0.1^{bc}	0.00	0.013	<0.001	<0.001
SCFA proportion, molar %														
Acetate	63.5^{d}	59.4^{g}	65.5^{c}	59.7^{g}	66.6^{bc}	59.8^{g}	67.7^{b}	61.6^{e}	69.5^{a}	63.6^{d}	0.49	<0.001	<0.001	<0.001
Propionate	20.6^{d}	25.5^{ab}	19.5^{de}	26.2^{a}	19.0^{ef}	26.6^{a}	18.5^{ef}	24.6^{b}	17.9^{f}	23.2^{c}	0.47	<0.001	<0.001	<0.001
iso-Butyrate	0.7^{cde}	0.7^{e}	0.8^{bcde}	0.7^{de}	0.8^{bcde}	0.9^{abcd}	0.9^{abc}	0.9^{ab}	0.9^{ab}	1.0^{a}	0.02	>0.999	<0.001	0.131
n-Butyrate	12.9^{a}	12.3^{ab}	11.9^{bc}	11.3^{cd}	11.1^{de}	10.6^{ef}	10.3^{f}	10.5^{f}	9.1^{g}	9.6^{g}	0.16	0.015	<0.001	<0.001
iso-Valerate	1.2^{bc}	1.1^{c}	1.3^{a}	1.1^{bc}	1.5^{abc}	1.2^{bc}	1.6^{a}	1.4^{abc}	1.5^{ab}	1.5^{abc}	0.04	0.007	<0.001	0.722
n-Valerate	1.1^{bc}	1.0^{e}	1.0^{bcd}	1.0^{e}	1.0^{cde}	1.0^{e}	1.0^{de}	1.1^{ab}	1.0^{de}	1.15^{a}	0.01	0.818	<0.001	<0.001
Ammonia mgN/100 mL	28.3^{b}	30.7^{ab}	31.3^{ab}	28.7^{b}	36.4^{a}	30.5^{ab}	36.9^{a}	33.3^{ab}	31.2^{ab}	31.7^{ab}	0.56	0.042	0.005	0.007

*Ginkgo extract (1.6% fruit equivalent) was added to treatment, while ethanol was to control.

Values in parenthesis are relative percentages of methane production in treatment to that in control.

[a-f]Means within a row with different superscripts significantly differ (P<0.05).

Table 9. Effect of ginkgo extract supplementation under different forage to concentrate ratios on rumen representative bacteria

Parameters	Gram reaction	Forage to concentrate ratios						SEM	*P*-value		
		1:9		5:5		9:1					
		Control*	Treatment*	Control	Treatment	Control	Treatment		Additive	Diet	A×D
Absolute abundance, log copies/mL culture											
Total bacteria	N/A	11.5^{a}	11.2^{b}	11.5^{a}	11.1^{bc}	11.5^{a}	11.0^{c}	0.041	<0.001	0.050	0.068
Relative abundance, %											
Total methanogen	N/A	1.71^{b}	1.72^{b}	2.11^{b}	1.86^{b}	1.63^{b}	3.25^{a}	0.122	0.050	0.020	<0.001
Butyrivibrio group	+	3.91^{b}	5.94^{a}	2.67^{cd}	3.36^{bc}	1.97^{d}	3.32^{bc}	0.240	<0.001	<0.001	0.240
Ruminococcus flavefaciens	+	0.01^{b}	0.01^{b}	0.02^{b}	0.01^{b}	0.04^{a}	0.01^{b}	0.002	<0.001	<0.001	<0.001
Ruminococcus albus	+	0.04^{bc}	0.02^{cd}	0.05^{bc}	0.01^{d}	0.11^{a}	0.05^{b}	0.006	<0.001	<0.001	0.079
Streptococcus bovis	+	0.14^{ab}	0.25^{a}	0.05^{b}	0.16^{ab}	0.02^{b}	0.08^{ab}	0.021	0.017	0.011	0.801
Treponema bryantii	-	0.03^{b}	0.01^{b}	0.02^{b}	0.01^{b}	0.07^{a}	0.05^{a}	0.005	<0.001	<0.001	0.870
Fibrobacter succinogenes	-	0.03^{a}	0.01^{c}	0.02^{b}	0.01^{c}	0.03^{a}	0.03^{ab}	0.002	<0.001	<0.001	0.010
Selenomonas ruminantium	-	0.34^{c}	1.40^{a}	0.18^{cd}	0.87^{b}	0.05^{d}	0.26^{bc}	0.089	<0.001	<0.001	<0.001
Prevotella ruminicola	-	1.31	0.89	0.86	1.08	0.96	1.03	0.052	0.669	0.547	0.037
Prevotella bryantii	-	0.03^{a}	0.03^{a}	0.02^{b}	0.01^{bc}	0.01^{a}	$>0.00^{a}$	0.002	0.439	<0.001	0.815
Anaerovibrio lipolytica	-	0.05^{bc}	0.15^{a}	0.02^{bc}	0.17^{a}	0.01^{c}	0.05^{b}	0.012	<0.001	<0.001	<0.001
Ruminobacter amylophilus	-	0.10^{b}	0.16^{b}	0.11^{b}	0.53^{a}	0.07^{b}	0.51^{a}	0.039	<0.001	<0.001	<0.001
Succinivibrio dextrinosolvens	-	0.21^{ab}	0.27^{a}	0.10^{bc}	0.32^{a}	0.04^{c}	0.30^{a}	0.023	<0.001	0.113	0.012
Megasphaera elsdenii	-	0.01^{b}	0.11^{a}	$>0.00^{b}$	0.11^{a}	$>0.00^{b}$	0.01^{b}	0.009	<0.001	<0.001	<0.001

*See Table 2.

N/A, not available.

[a-d]Means within a row with different superscripts significantly differ (P<0.05).

3.4. Discussion

3.4.1. Rumen fermentation

In the present study, changes in rumen fermentation with ginkgo extract supplementation of diets with different F:C ratios were investigated. The nutritive value of the experimental substrates varied depending on the F:C ratio, as shown for the levels of CP (87–190 g/kg) and NDF (385–702 g/kg) (Table 7). We employed various F:C ratios to simulate a variety of feeding regimens for ruminant animals in accordance with growth stage and production purpose (e.g., for fattening or dairying). Therefore, the present evaluation might be useful in the preliminary screening of dietary conditions under which ginkgo extract shows significantly favorable effects.

The extent of methane reduction by ginkgo extract was dependent on the F:C ratio; the 5:5 ratio followed by the 7:3 ratio showed remarkable reductions (Table 8). However, methane production was not clearly affected by the F:C ratio alone, which is in agreement with that reported by (Goiri et al., 2009). Hydrogen accumulation was observed only for the ginkgo extract treatment (Table 8). Hydrogen released from hydrogen-producing rumen microbes (Zhou et al., 2012), is metabolized in an alternative pathway to methanogenesis when methane production is inhibited (Mitsumori et al., 2012). One such pathway involves fumarate reduction, which leads to propionate production (Newbold et al., 2005). If propionate is enhanced to metabolize hydrogen, it could lead to greater efficiency of feed energy utilization (Ungerfeld, 2015).

3.4.2. Rumen bacteria

Dietary ginkgo extract supplementation at various F:C ratios might cause alterations in rumen fermentation (e.g., higher propionate and lower methane production) as a result of microbial

selection (Oh et al., 2017). The largest bacterial increase related to propionate production (*S. ruminantium, A. lipolytica* and *M. elsdenii*) following ginkgo extract supplementation was observed in the 5:5 substrate (Table 9). This may explain why the 5:5 ratio diet resulted in the largest methane reduction (41.9%, Table 8). In addition to these species, *R. amylophilus* and *S. dextrinosolvens* are known to contribute to propionate production (O'Herrin and Kenealy, 1993; Stackebrandt and Hespell, 2006). However, these two species did not show clear response to ginkgo extract supplementation. This may be the reason for the least methane reduction with the 1:9 ratio (14.6%, Table 8). They are tolerant to low pH led by the high concentrate diet (Patterson and Hespell, 1985; Stackebrandt and Hespell, 2006) and might have already reached the maximum level under the condition tested.

The effect of dietary ginkgo extract supplementation on rumen ammonia was not clear (Table 8). Chapter 2 describes that ammonia is decreased by ginkgo extract in the artificial rumen, possibly by inhibiting hyper-ammonia producing bacteria such as *Peptostreptcoccus anaerobious, Clostridium aminophilum, Clostridium sticklandii* (Chen and Russell, 1989). The present short-term incubation may not affect those bacteria in a similar manner, though they were not quantified in the present study.

Ginkgo extract supplementation reduced the levels of *R. albus* and *R. flavefaciens* under a high forage diet condition (Table 9), possibly leading to decreased acetate production (Table 8). These bacteria were previously found to be reduced by both ginkgo extract and anacardic acid as its main active compound in a pure culture study (Oh et al., 2017). Inhibition of these hydrogen-producing bacteria to levels that do not negatively affect rumen fermentation is thought to be an effective strategy to reduce methane production (Morgavi et al., 2010). Although abundance of

specific bacteria discussed above is quite low at DNA level in the present study (0.1-1.40%), changes of their metabolic activity might be influential in fermentation parameters. Therefore, transcriptomic and proteomic analyses are clues for deeper discussion.

3.5. Conclusion

The ginkgo fruit extract showed positive effects on rumen modulation, i.e., less methane and more propionate production under various diets, with no adverse impact on feed fermentation ability as indicated by total SCFA production. For application of ginkgo extract as a feed additive for ruminant animals, additional practical evaluations are required, such as long-term cultivation studies and animal feeing trials under a wide range of dietary conditions.

Chapter 4. General discussion and perspective

4.1. Antimicrobial property of ginkgo

Anacardic acid in ginkgo, accounting for 85% of alkylphenolic compounds, was found to be a major compound to inhibit specific group of rumen bacteria. Although the alkyl side-chain structure is different (C13:0, C15:1 and C17:1), these anacardic acids in ginkgo were potent for selective inhibition of rumen bacteria (Table 6). Length of alkyl side chain is known to be important factor in antimicrobial activity (Kubo et al., 1993), while number of double bond in alkyl side chain is not an essential matter (Kubo et al., 1999). This suggests that ginkgo fruit is as potent as CNSL in altering rumen microbial community via the surfactant action of anacardic acid (Kubo et al., 1993) to lead fermentation changes, if dosed at a suitable level (Watanabe et al., 2010; Shinkai et al., 2012). Fruit of ginkgo contains a greater proportion of anacardic acids than other parts (leaves or seeds) that have been commercially used (van Beek and Montoro, 2009). Therefore, the fruit is considered more functional as an antimicrobial and more easily available in terms of quantity than leaf and seed.

Other bioactive compounds besides alkylphenols in ginkgo extract such as flavonoid might influence rumen fermentation to decrease rumen methane (Kim et al., 2015). Ginkgo leaf polysaccharides possesses antioxidant activity (Jiao et al., 2016) which also affects rumen fermentation. Unlikely to CNSL having simple alkylphenols, synergistic action between these functional compounds in ginkgo should be confirmed. Muroi et al. (2004) reported that anacardic acid and methicillin showed an interaction to inhibit methicillin resistant *Staphylococcus aureus* by which minimum inhibitory concentration of anacardic acid varied 5-32 folds. Although, more details on the mode of action of ginkgo extract is experimentally to be investigated, alkylphenols

represented by anacardic acid could be a main factor in altering rumen microbiota and fermentation.

4.2. Rumen fermentation and feed digestion

Ginkgo fruit extract altered rumen fermentation with no adverse effect on SCFA level, although extent of methane reduction was diet-dependent (Table 8). Hydrogen accumulation was observed only with ginkgo supplementation, indicating that hydrogen released from hydrogen-producing bacteria is not completely well metabolized via alternative pathways involved in propionate production rather than methanogenesis. Fumarate reduction pathway leading propionate production is the best alternative pathway which would need adaptation period to be developed (Newbold et al., 2005). If the pathway is fully developed and propionate is enhanced to metabolize hydrogen, a greater efficiency of feed energy utilization could be expected (Ungerfeld, 2015). SCFA composition was greatly changed by ginkgo extract toward less acetate and more propionate production. Such great shift in rumen fermentation pattern is attributed to rumen microbial changes by selective inhibition of rumen microbes with ginkgo supplementation.

Ammonia reduction within a critical level (>5mgN/dL) by ginkgo fruit extract might suggest better dietary N economy for maintaining microbial protein synthesis (Griswold et al., 2003). This reduction could be related to inhibition of hyper-ammonia producing bacteria *C. stickandii* and *P. anaerobius* by ginkgo extract, i.e. these species were confirmed quite sensitive to ginkgo extract when those were individually cultivated (Table 6). Population size of protozoa is also related to ruminal ammonia level (Patra and Yu, 2014). The RUSITEC study showed significant reduction of rumen ammonia (Table 4), though batch culture study did not (Table 8). The disappearance of DM, NDF, and ADF were not affected by ginkgo extract in RUSITEC study

(table 4). This is the most important characteristic to be addressed for developing a new additive candidate, as reported for CNSL (Watanabe et al., 2010).

Overall, rumen fermentation pattern was more ideally changed in RUSITEC (7 d incubation) than batch culture studies (24 h incubation), suggesting that fermentation shift needs a certain time period for adaptation to the additive applied. Therefore, longer incubation or feeding studies are obviously needed for more practical evaluation.

4.3. Rumen microbial changes

Rumen fermentation changes by ginkgo extract were reasonably attributed to microbial community changes that were evaluated by qPCR (figure 1) and MiSeq (Figure 2) analyses, and also by sensitivity of individual rumen bacterial species to the extract (Table 6). Microbial selection by ginkgo extract was suggested by growth inhibition of hydrogen- and formate-producing bacteria (*R. flavefaciens* and *R. albus*) and by growth stimulation of succinate- and/or propionate-producing bacteria (*P. ruminicola*, *S. dextrinosolvens*, *R. amylophilus*, *S. ruminantium*, and *M. elsdenii*) according to qPCR and MIC results. Batch culture study using different dietary condition indicated that the largest bacterial increase relating propionate production (*S. ruminantium, A. lipolytica* and *M. elsdenii*) following ginkgo extract supplementation was observed with 5:5 in F:C ratio (Table 9). In addition to these species, *R. amylophilus* and *S. dextrinosolvens* did not show clear response to ginkgo extract supplementation. They are tolerant to low pH led by the high concentrate diet (Patterson and Hespell, 1985; Stackebrandt and Hespell, 2006) and might have already reached the maximum level under the condition tested. Although butyrate producers (*E. ruminantium*, *B. fibrisolvens*, and *B. proteoclasticus*) were quite sensitive to ginkgo extract, as

seen by the MIC, butyrate production in batch cultures or RUSITEC was not decreased by ginkgo extract supplementation. This is due likely to the increased abundance of *M. elsdenii* by ginkgo extract, which also produces butyrate.

According to the qPCR observation in RUSITEC study (Figure 1), decreased number of protozoa and fungi, which are hydrogen and formate producers, respectively, is also indicative of these fermentation changes. Although the abundance of fibrolytic organisms (*Fibrobacter*, *Ruminococcus*, fungi) was depressed by ginkgo extract supplementation, NDF and ADF digestibility was not affected (Table 4). This suggests development of an alternative fibrolytic system, possibly due to an increase in abundance of *Prevotella* and *Selenomonas* (Figure 2), some groups of which are involved in fiber degradation (Bekele et al., 2010; Sawanon et al., 2011). The abundance of *P. ruminicola* was not affected by F:C ratio, supporting that this bacterium might play an important role in digestion of a wide range of diet. The contribution of uncharacterized fibrolytic bacteria (Kobayashi, 2006; Jami et al., 2012) may also need to be considered for deeper discussion about fiber digestion. Although reduced protozoa and fungi may cause the decrease of fiber degradation, protozoa maintained in RUSITEC were non-fibrolytic entodinia (Béra-Maillet et al., 2005), and fungi might have already been selected for less fibrolytic ones in the 70% concentrate diet used in RUSITEC study.

Decreased abundance of total methanogens, protozoa, and fungi by ginkgo extract indicates both direct and indirect inhibition of methanogenesis, because both of planktonic methanogens and eukaryote (protozoa and fungi)–associated methanogens (Vogels et al., 1980; Cheng et al., 2009) are involved in methane synthesis. This indicates the occurrence of archaeal selection via inhibition of protozoa and fungi by ginkgo.

4.4. Perspectives

In application, one problem needs to be solved: ginkgo fruit contains nutritionally harmful 4-methylpyridoxin (a vitamin B_6 antagonist) (Isah, 2015), which may be unsuitable for animal feed, leading to deficiency of vitamin B_6 for animals. If the activity of this antinutritional compound can be reduced by specific treatments, ginkgo fruit can be further evaluated in animal feed studies. One solution could be silage fermentation. Some lactic acid bacteria can produce vitamin B_6 (Champagne et al., 2010), which might compensate for the negative effects of 4-methylpyridoxin if those bacteria are used as a silage inoculant. This possibility needs to be confirmed to better assess the functionality of ginkgo fruit as a potential feed additive for ruminant animals.

Although beneficial effects of antibiotics on rumen modulation and animal performance are not ignorable, the use of antibiotics might be shrunk in the future, because strict laws concerning public health have been established (McGuffey et al., 2001; Beauchemin et al., 2008). Functional phytogenic compounds and their source plants can be an alternative to antibiotics that have been used to animals for a long period of time. Plant produces a variety of metabolite, some of which often exhibit beneficial functions such as antimicrobial (Kubo et al., 1993) and antioxidative (Kubo et al., 2006) activity. These are thought to be helpful for the promotion of animal health and production. Reducing ruminal methane and fecal nitrogen with such supplement might be helpful for solving environmental issues. Attempts to screen such functional materials and detailed evaluation of those in application to animal industry should be made further.

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