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Ginkgo fruit extract as an additive to modify rumen microbiota and fermentation and to mitigate methane production

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ABSTRACT

Ginkgo fruit, an unused byproduct of the ginkgo nut industry, contains antimicrobial compounds known as anacardic acids. Two major cultivars of ginkgo, Kyuju (K) and Tokuro (T), were evaluated for their potential as a feed additive for ruminants. In batch culture, we incubated a mixture of hay and concentrate in diluted rumen fluid with or without 1.6% (fruit equivalent) ginkgo fruit extract. We conducted another series of batch culture studies to determine the dose response of fermentation. We also conducted continuous culture using the rumen simulation technique (RUSITEC) with cultivar K and carried out a pure culture study to monitor the sensitivity of 17 representative rumen bacterial species to ginkgo extract and component phenolics. Although both K and T extracts led to decreased methane and increased propionate production, changes were more apparent with K extract, and were dose-dependent. Total gas production was depressed at doses ≥3.2%, suggesting that 1.6% was the optimal supplementation level. In RUSITEC fermentation supplemented with 1.6% ginkgo K, methane decreased by 53% without affecting total gas or total VFA production, but with decreased acetate and increased propionate. Disappearance of dry matter, neutral detergent fiber, and acid detergent fiber were not affected by ginkgo, but ammonia levels were decreased. Quantitative PCR indicated that the abundance of protozoa, fungi, methanogens, and bacteria related to hydrogen and formate production decreased, but the abundance of bacteria related to propionate production increased. MiSeq analysis (Illumina Inc., San Diego, CA) confirmed these bacterial changes and identified archaeal community changes, including a decrease in *Methanobrevibacter* and *Methanomassiliicoccaceae* and an increase in *Methanoplanus.* Pure culture study results supported the findings for the above bacterial community changes. These results demonstrate that ginkgo fruit can modulate rumen fermentation toward methane mitigation and propionate enhancement via microbial selection.

Key words: ginkgo fruit extract, methane reduction, rumen fermentation, rumen microbiota

INTRODUCTION

Ginkgo (*Ginkgo biloba*) is a so-called living fossil classified as a deciduous tree. Ginkgo is widely distributed in Asian countries, including China, Japan, and Korea. In these areas, ginkgo nuts are consumed as food and as medicine. In Japan, about 1,000 t/yr of ginkgo nuts are produced from 6 cultivars, which differ by harvest period and the shape and size of the nut. Ginkgo fruit is a byproduct of ginkgo nut production, but is not used due to its peculiar smell and the presence of nutritionally negative compounds, such as a vitamin B_6 antagonist. Recently, however, interest has been growing in the functional compounds (such as flavonoids, terpene trilactones, and alkylphenols) present in ginkgo leaves (van Beek and Montoro, 2009) and ginkgo fruit (Yang et al., 2004). Of these biochemical groups, the alkylphenols, represented by anacardic acid, cardanol, and cardol, have been found to possess useful characteristics such as antitumor (Itokawa et al., 1987), antifeedant (Matsumoto and Sei, 1987), antistress (Rai et al., 2003), and antibacterial (Kubo et al., 1993) activities. More recently, studies using ginkgo fruit and leaves have assessed their use as a feed additive for broiler chickens (Zhang et al., 2012) and piglets (Zhou et al., 2015). Cashew nut shell liquid (**CNSL**), which contains similar alkylphenols, 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), but anacardic acids in ginkgo consist of 3 molecules with different side-chain lengths (C13:0, C15:1, and C17:1). A previous report suggested that the length of the alkyl side chain of anacardic acid differentially affects antibacterial activ-

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ity 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. We hypothesized that ginkgo fruit containing anacardic acid can modulate rumen fermentation without negatively affecting feed digestion. Therefore, we investigated rumen parameters, including gas, VFA, ammonia, and microbes, to evaluate the potential of gingko fruit extract as a rumen modifier.

MATERIALS AND METHODS

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.

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.

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 orchardgrass 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 VFA 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).

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.