Effects of micronised wood powder irradiated with ultraviolet light and exposed to ozone gas on *in vitro* ruminal fermentation in beef cattle

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Abstract

In *in vitro* ruminal fermentation, micronised wood powder is digested to produce volatile fatty acids, but its fermentability is weak. The aim of the present study was to ascertain a suitable pretreatment of micronised wood powder to enhance volatile fatty acid production *in vitro*. Rumen fluid taken from beef cattle was anaerobically incubated with micronised wood powder. Initially, effects of particle size of micronised wood powder on ruminal fermentation were examined. Volatile fatty acid production was then evaluated by slightly reducing methanol-soluble substances such as lignin in micronised wood powder. Further, micronised wood powder that had been irradiated by an ozone lamp (185 and 254 nm) or sterilisation lamp (254 nm) was fermented in rumen fluid. The lack of volatile fatty acid production by rumen fermentation of untreated micronised wood powder was not associated with its particle size. In the methanol-treated micronised wood powder, volatile fatty acid production was increased, and this response was sensitively dose-dependent. Moreover, pretreatment by ozone lamp irradiation stimulated micronised wood powder fermentation and resulted in increased volatile fatty acid concentrations, especially of acetate and propionate. The response to ozone-treatment of micronised wood powder increased propionate more than acetate. In addition, this pretreatment of micronised wood powder to improve rumen fermentation was superior to that with a sterilisation lamp (254 nm). Therefore, the ruminal fermentability of micronised wood powder was not dependent on its particle size. The present study suggested that irradiation by ozone lamp is useful to enhance micronised wood powder digestibility and that this treated powder may become a lignified biomass feedstuff capable of volatile fatty acid production in ruminants.

Introduction

Recently, the bioethanol and biogas production of lignocellulosic biomasses has been tested in order to utilise its polysaccharides, cellulose and hemicellulose [1]. In animal production, sawdust feeding has been examined as roughage to maintain desirable rumen function [2,3]. Previous studies have found that body weight gain improved in sawdust-fed cattle [4,5]. However, ruminants would have difficulty digesting lignified holocelluloses directly because El-Sabban *et al.* [3] has reported that long-term feeding of oak sawdust did not affect the ruminal volatile fatty acid profile in steers. In plant cell walls, lignin is covalently bound to hemicelluloses and gradually develops the strength and rigidity of cell walls [6,7]. In ruminal fermentation, cellulase activity caused by bacteria and fungi [8] is affected by cross-linkages of lignin [9]. Thus, especially in woody feedstuffs, the cross-linkage of lignin will interfere with the ruminal anaerobic digestion (fermentation) of lignocelluloses for volatile fatty acid production.

Many technical methods have been considered and developed to utilise lignocellulosic biomasses for bioethanol production. Steam explosion and alkaline oxidative delignification (hydrogen peroxide) have been effective in solubilisation of hemicellulose and removal of lignin [10-12]. Rangnekar *et al.* [13] reported that high-pressure steam treatment enhanced *in vitro* ruminal digestion of straw and bagasse. In addition, use of white-rot fungi capable of lignin degradation has been reported to improve the digestibility of lignocellulose *in vitro*

[14,15]. These pretreatments for enhancing holocellulose digestibility are, however, carried out under wet conditions and/or in an aqueous solution. If ruminants can digest dry wood lignocelluloses directly for volatile fatty acid production, this biomass would be easy to use as a useful feedstuff. More recently, Takahashi *et al.* [16] developed the tandem-ring mill, a pulverizing device, capable of manufacturing micronised wood powder. It was found previously that micronised wood powder can be directly fermented to increase the volatile fatty acid production of rumen fluid *in vitro*, but the degree of this fermentation was small [17]. The lack of ruminal fermentation of micronised wood powder may be associated with interference with the cellulolytic activity of rumen microorganisms by lignin in relation to its particle size. However, even in fine wood powder, it still remains uncertain whether the lignin in micronised wood powder interferes with cellulolytic activity in microbial fermentation.

On the other hand, a photochemical reaction using ultraviolet (UV)

light is available to decompose lignin [18,19]. In addition, ozone, which is a powerful oxidant, can break down lignin [20,21]. Photochemical pretreatment with a UV lamp producing ozone gas (ozone lamp) that has been used in wastewater treatment [22] may also be effective to decompose lignin, which is responsible for improved volatile fatty acid production, for use of micronised wood powder as a feedstuff. The characteristic powder form of micronised wood powder would possess an advantage in that the powdered wood lignocelluloses capable of being mixed can be exposed uniformly to UV light and react deeply with ozone gas.

Therefore, the aim of the present study was to ascertain whether pretreatment of micronised wood powder improves its ruminal fermentability for volatile fatty acid production *in vitro*. Initially, the effect of micronised wood powder particle size on ruminal fermentation was examined. In addition, whether volatile fatty acid production was increased when the methanol-soluble substances including lignin were slightly reduced in micronised wood powder was evaluated. Finally, the present study determined the effect of irradiation of micronised wood powder with an ozone lamp on rumen fermentation.

Materials and methods

The present study was approved by the Animal Care and Use Committee at Akita Prefectural University. This study consisted of four experiments to evaluate *in vitro* ruminal fermentation of micronised wood powder for volatile fatty acid production using batch incubation of rumen fluid. The ruminal fermentation in relation to particle size of micronised wood powder was examined in Experiment 1. In Experiment 2, removal of methanol-soluble substances that stimulate volatile fatty acid production derived from its lignocelluloses was evaluated. Experiments 3 and 4 determined whether micronised wood powder irradiated with UV light improves its fermentation for volatile fatty acid production.

Preparation of micronised wood powder

For preparation of micronised wood powder, wood chips of Japanese cedar (*Cryptomeria japonica* D. Don) sapwood were pulverised using a tandem-ring mill in accordance with the methods of Takahashi *et al.* [16]. Concentrations of cellulose, hemicellulose, and lignin in micronised wood powder (air-dried basis) were 41.2%, 16.1%, and 30.0%, respectively. To prepare micronised wood powder classified by three different particle diameters, the wood powder was passed through sieves of 100, 53, and 38 μm diameters. The micronised wood powder particles less than 38 μm in diameter were classified as small particle size, those exceeding 38 μm and less than 53 μm diameter as medium particle size, and those exceeding 53 μm and less than 100 μm diameters as large particle size. All three sizes of micronised wood powder were used for Experiment 1. In addition, micronised wood powder particles less than 100 μm in diameter were used for Experiment 2. In Experiments 3 and 4, micronised wood powder particles less than 53 μm were irradiated by UV lamps.

In Experiment 2, the present study prepared methanol-treated micronised wood powder from which lignin was crudely removed because lignin is partially soluble in methanol [23]. This solubilisation with methanol was performed at room temperature to remove methanol-soluble compounds partially and slightly from micronised wood powder. Methanol (200 ml) was added to 10g micronised wood powder, and this mixture was stirred for 120 min. Thereafter, micronised wood powder was filtered through a No. 5A filter paper and washed several times with additional methanol. micronised wood

powder was then dried initially at 40°C and subsequently at 70°C for 5 hours each. Finally, micronised wood powder was stored at room temperature. Air-dried methanol-treated micronised wood powder was used as a substrate for *in vitro* ruminal fermentation.

For Experiments 3 and 4, micronised wood powder (10 g) was transferred into a stainless-steel tray and spread to a layer less than approximately 0.5 mm. The tray was placed in a plastic box lined with aluminum foil. The box was closed with a cover that had a UV lamp attached inside. Thereafter, micronised wood powder was continuously irradiated by either an ozone lamp or a sterilisation lamp. Micronised wood powder irradiated by an ozone lamp (GL10ZH, 10W; Sankyo Denki Co., Kanagawa, Japan) was used for Experiments 3 and 4. The major wavelengths of the ozone lamp were of 254 nm and 185 nm. The light irradiation lasted for 21 days, and the thin layer of micronised wood powder was mixed manually twice daily. The ozone gas concentration in the box measured with a gas test tube (Gastec Co., Kanagawa, Japan) was 22 μl/l during irradiation with an ozone lamp.

Animals and diets

A Japanese Shorthorn steer (495 kg; 21 months old), fed hay consisting of orchard grass and perennial ryegrass *ad libitum* and 1.5 kg of concentrate mixture containing soybean powder, wheat, and rice bran twice daily at 900 and 1600, was used as the donor animal for Experiments 1–3. In Experiment 4, five Japanese Shorthorn beef heifers (483 ± 28 kg; 22–23 months old) fed hay and the micronised wood powder concentrate mixture, similar to the steer used in Experiment 1, were used as donors of rumen fluid for the batch fermentation.

In vitro batch fermentation of rumen fluid

About 600 ml of ruminal fluid from donor animals was obtained 5–6 hours post-morning feeding using a stainless steel oral catheter for cattle (Sanshin Industrial Co. Ltd., Kanagawa, Japan) and a vacuum pump. The inside of the inner tube and the sampling bottle were filled with O_2-free CO_2 gas prior to sampling. After sampling, the bottle was anaerobically and immediately warmed in a portable incubator (39 ± 1°C). Ruminal fluid was filtered through four layers of surgical gauze into a polypropylene beaker under flushing with O_2-free CO_2 gas. The rumen fluid (10 ml) was immediately mixed with buffer (pH 6.8, 20 ml) as artificial saliva (292 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_2O$, 4,000 mg Na_2CO_3, and 600 mg cysteine hydrochloride) in accordance with the method of Lila *et al.* [24] in a 50 ml polypropylene test tube containing the micronised wood powder substrate. The tubes had been warmed in metal beads (Lab Armor Beads, Lab Armor LLC, OR. USA) set at 39°C beforehand. Duplicate samples were screw-capped and further sealed with airtight tape after CO_2 flushing. The mixed rumen fluid was anaerobically incubated at 39°C for 24 hours (Experiments 1–3) or 48 hours (Experiment 4) using an air-circulating incubator. Shaking of the samples during the incubation was performed using a twist-rotating shaker (about 40 rpm).

In Experiment 1, three different particle sizes of micronised wood powder (200 mg per tube) were added as a substrate (n=5). In controls, the only mixed ruminal fluid was incubated. In Experiment 2, different levels of micronised wood powder treated with methanol at 0 (control), 200, 300, or 400 mg per tube (n=5 each) were added to duplicate samples. Furthermore, micronised wood powder irradiated with UV light was fermented in the mixed rumen fluids at the levels of 200 and 400 mg per tube in Experiments 3 and 4 as a factorial arrangement (n=5). Duplicate samples supplemented with the original micronised

wood powder untreated with methanol or UV lights were prepared and used as controls (n=5). After incubation, pH was immediately measured and a portion of rumen fluid was used to assay volatile fatty acid concentrations.

Determinations of pH value and volatile fatty acid concentration

The pH values of the incubated ruminal fluid were measured with a pH meter (model 827 pH lab, Metrohm AG, Herisau, Switzerland). For the volatile fatty acid determination, mixed incubated rumen fluids were centrifuged for 10 min at 4°C (9,000 *g*). A portion (1.0 ml) was mixed with 200 μl of a 25% metaphosphoric acid–formic acid mixture (3:1) [25]. Thereafter, 100 μl of an internal standard (20 μmol pivalic acid; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was added to the samples. The mixture was incubated for 30 min at 4°C and centrifuged (9,000 *g*) for 10 min at 4°C. The supernatant was used for gas chromatography. Volatile fatty acids were determined using a gas chromatography system (model GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame ionisation detector, an auto-injector (AOC-20i, Shimadzu), and a fused silica capillary column (Nukol, 30 m × 0.25 mm × 0.25 μm, Supelco, PA. USA). Helium gas was used as the carrier gas at a flow rate of 1.15 ml/min. The temperature of the injector and detector was set at 250°C. The temperature in the column oven was initially set at 90°C, then linearly increased to 185°C at a rate of 10°C/min, and held for 2.5 min. The injection volume of a sample was 1 μl, and the split ratio was 70:1. Short chain fatty acids were identified and quantified by their retention times and peak area with standards and internal standard (Tokyo Chemical Industry Co., Ltd.) using a computer program for data analysis (GC solution, Shimadzu).

Statistical analysis

Statistical analysis for analysis of variance (ANOVA) was performed using a computer program (StatView, Abacus Concepts Inc., CA, USA). In Experiments 1 and 2, data on pH values and volatile fatty acid concentrations were analysed by one-way ANOVA. When a significant effect of micronised wood powder was detected by the ANOVA, Tukey's honestly significant difference test was used to determine the significance of responses to micronised wood powder supplementation (KaleidaGraph, Synergy Software, PA, USA). In Experiments 3 and 4, volatile fatty acid concentrations were statistically analysed by two-way ANOVA (micronised wood powder treatments × its supplemented levels). If a significant effect on a volatile fatty acid concentration was detected as a main factor of micronised wood

powder treatment (Experiment 3) or interaction between micronised wood powder treatment and its supplemented level, significant differences were analysed using Tukey's honestly significant difference test for comparing treatment groups.

Results

Experiment 1: Effects of particle size of micronised wood powder on *in vitro* ruminal fermentation

This experiment evaluated whether ruminal fermentation of micronised wood powder is dependent on its particle size. Table 1 shows alterations of pH value and volatile fatty acid concentrations after batch fermentation of micronised wood powder divided into three classes by particle size. Compared with the control, micronised wood powder slightly decreased (P<0.05) pH values, independently of particle size. Concentrations of total volatile fatty acid in micronised wood powder-supplemented groups were higher (P<0.05) than that in controls regardless of its particle classes. The increased total volatile fatty acid concentrations were due to stimulated production of the major volatile fatty acids, acetate, propionate, and butyrate (P<0.05). Consequently, the particle size of micronised wood powder did not affect these short chain fatty acid productions. In addition, the molar ratio of acetate to propionate remained constant. In minor volatile fatty acids, a slight increase in short chain fatty acid concentration occurred in isovalerate in the groups supplemented with different particle sizes of small and medium range of micronised wood powder (P<0.05). Thus, these results indicated that particle size of micronised wood powder was not an important factor limiting its ruminal fermentation only if the size is smaller than 100 μm. However, the extent of volatile fatty acid production due to micronised wood powder supplementation remained constant.

Experiment 2: Ruminal fermentation of micronised wood powder treated with methanol

Table 2 shows changes in pH values and volatile fatty acid production with *in vitro* ruminal fermentation of methanol-treated micronised wood powder. A significant reduction in pH value was observed in the methanol-treated micronised wood powder group compared with controls (P<0.05). This response was greater in the groups supplemented with the methanol-treated micronised wood powder at 300 and 400 mg than at 200 mg. In addition, total volatile fatty acid concentrations increased in the groups supplemented with the methanol-treated micronised wood powder. Greater responses of total

Table 1. Effects of different particle size of micronised wood powder on *in vitro* ruminal fermentation for 24 hours (Experiment 1).

Treatment		Particle size of micronised wood powder				ANOVA (*P*-value)
	Control	Small	Medium	Large	SEM	
pH	6.47[a]	6.34[b]	6.36[b]	6.37[b]	0.012	< 0.001
			mmol/l			
Total volatile fatty acids	32.0[b]	37.6[a]	36.7[a]	36.1[a]	0.60	< 0.001
Acetate	23.2[b]	27.0[a]	26.4[a]	26.1[a]	0.40	< 0.001
Propionate	4.52[b]	5.16[a]	5.02[a]	5.01[a]	0.071	0.0013
Butyrate	3.62[b]	4.44[a]	4.34[a]	4.09[a]	0.087	< 0.001
Isobutyrate	0.17	0.22	0.21	0.20	0.007	0.0556
Valerate	0.12	0.12	0.13	0.13	0.016	0.1338
Isovalerate	0.32[b]	0.47[a]	0.44[a]	0.40[ab]	0.003	0.0041
Capronate	0.08	0.14	0.13	0.11	0.010	0.1503
Acetate / Propionate ratio	5.13	5.24	5.27	5.22	0.030	0.4947

Values represent means with standard error of means (n=5).
[a,b]Mean values within a row not sharing a common superscript letter were significantly different, P<0.05.

Table 2. Effects of micronised wood powder treated with methanol on *in vitro* ruminal fermentation for 24 hours (Experiment 2).

Treatment	Micronised wood powder treated with methanol[1] (mg)					ANOVA (*P*-value)
	0	**200**	**300**	**400**	**SEM**	
pH	6.62[a]	6.56[b]	6.41[c]	6.42[c]	0.024	< 0.001
	mmol/l					
Total volatile fatty acids	40.3[c]	44.0[b]	46.4[a]	48.8[a]	0.76	< 0.001
Acetate	28.4[c]	30.9[b]	32.5[a]	34.0[a]	0.52	< 0.001
Propionate	5.65[c]	6.46[b]	7.02[a]	7.50[a]	0.168	< 0.001
Butyrate	4.73[b]	5.02[ab]	5.19[a]	5.37[a]	0.068	< 0.001
Isobutyrate	0.32	0.34	0.35	0.35	0.006	0.2231
Valerate	0.51[b]	0.57[ab]	0.56[ab]	0.59[a]	0.011	0.0225
Isovalerate	0.47	0.46	0.45	0.45	0.025	0.7004
Capronate	0.29	0.31	0.33	0.31	0.008	0.3420
Acetate / Propionate ratio	5.02[a]	4.78[ab]	4.62[b]	4.53[b]	0.057	0.0026

Values represent means with standard error of means (n=5).
[1]Fine particle size less than 53 μm diameters was used.
[a-c]Mean values within a row not sharing a common superscript letter were significantly different, P<0.05.

volatile fatty acids to the methanol-treated micronised wood powder occurred at 300 and 400 mg compared with the supplementation level of 200 mg. A similar significant effect was shown in concentrations of the major short chain fatty acids of acetate and propionate (P<0.05) but not in butyrate concentrations. However, an increase in butyrate concentration was observed in the groups of methanol-treated micronised wood powder at 300 and 400 mg. The ratio of acetate to propionate was lower (P<0.05), compared with controls, when the supplementation level of the methanol-treated micronised wood powder was enhanced (300 and 400 mg). In minor volatile fatty acids, significant response to the methanol-treated micronised wood powder occurred only in valerate (P<0.05). An increased valerate concentration was shown at the highest supplementation level of the methanol-treated micronised wood powder, compared with control (P<0.05). Overall, the response of volatile fatty acid production to the methanol-treated micronised wood powder was sensitively dosage-dependent. Hence, ruminal fermentation of micronised wood powder was improved when the methanol-soluble wood substances such as lignin were removed from micronised wood powder.

Experiment 3: Ruminal fermentation of micronised wood powder irradiated with ozone lamp

The results of ruminal fermentation of micronised wood powder with an ozone lamp are shown in Table 3. Compared with the group supplemented with untreated micronised wood powder as a control, the ozone lamp treatment was apparently effective for increasing micronised wood powder fermentation. Total volatile fatty acid concentrations in ozone-treated micronised wood powder groups were higher (P<0.001) than in controls, while there was no significant interaction between micronised wood powder treatment and its dosage. A similar response was observed in acetate concentrations (P<0.01). In propionate concentrations, a significant interaction between micronised wood powder treatment and micronised wood powder dosage was indicated (P<0.01). Irradiation of micronised wood powder with an ozone lamp stimulated propionate production, compared with controls. In addition, this effect was dosage-dependent in the groups of ozone-treated micronised wood powder (P<0.05), but not in controls supplemented with untreated micronised wood powder. Irradiation of micronised wood powder with an ozone lamp led to increased propionate concentration and consequently lowered (P<0.05) the ratio of acetate to propionate when the dosage of ozone-treated micronised

wood powder increased. However, the butyrate concentration remained constant. In minor volatile fatty acids, concentrations of valerate and capronate increased slightly in the ozone-treated micronised wood powder group (P<0.01). Thus, the ruminal fermentation of micronised wood powder for volatile fatty acid production was clearly improved by pretreatment of ozone lamp radiation.

Experiment 4: Ruminal fermentation of different micronised wood powder irradiated with UV and ozone lamps

In this experiment, ruminal fermentation was estimated by changes in volatile fatty acid concentrations as the difference from the values of the mixed rumen fluid alone without micronised wood powder supplementation because there were great variations in blank (basal) volatile fatty acid concentrations between individual animals. Overall, there was no significant interaction between UV-light treatments and their dosage. The total volatile fatty acid concentration, including minor short chain fatty acids, was significantly increased (P<0.05) by the ozone lamp-irradiated micronised wood powder, compared with controls that were supplemented with the original micronised wood powder. In addition, the major short chain fatty acids leading to increased total volatile fatty acid concentrations were acetate (P<0.05) and propionate (P<0.001) but not butyrate. However, micronised wood powder irradiated with a sterilisation lamp (UV light at 254 nm) had no effect on the concentration of total volatile fatty acids or major short chain fatty acids. Thus, micronised wood powder treated with an ozone lamp was superior to that treated with a sterilisation lamp for stimulating volatile fatty acid production in rumen fermentation.

Discussion

Among ligneous resources, feeding sawdust has been reported to improve weight gain of steers and lambs [4], and heifers [26]. However, El-Sabban *et al.* [3] reported that oak sawdust did not contribute to volatile fatty acid production in rumen fluid of steers *in vivo* because sawdust has a greater particle size relative to micronised wood powder. Sawdust will indirectly play a role as a roughage substitute for preventing abnormality of the gastrointestinal tract [3,5]. In the plant cell wall, holocelluloses are protected by lignin [27]. The lack of fermentability of woody lignocelluloses would be attributable to particle size, as in sawdust, or cross-linkage of lignin. In this study, it was confirmed that the fine wood powder could contribute to volatile fatty acid production in *in vitro* ruminal fermentation. This effect was

Table 3. Effects of ozone light-irradiated micronised wood powder (MWP) on *in vitro* ruminal fermentation for 24 hours (Experiment 3).

	MWP (mg)	Ozone lamp irradiation		Mean	SEM	ANOVA (P-value)		
		-[1]	+			Ozone	Amounts	Interaction
		mmol/l						
Total volatile fatty acids	200	40.4	43.2	41.8	0.66	< 0.001	0.0039	0.1501
	400	41.8	46.6	44.2				
	Mean	41.1	44.9					
Acetate	200	28.6	30.2	29.4	0.38	0.0012	0.0355	0.3008
	400	29.3	31.8	30.5				
	Mean	28.9	31.0					
Propionate	200	6.82[c]	7.57[b]	7.20	0.240	< 0.001	< 0.001	0.0030
	400	7.06[bc]	9.09[a]	8.07				
	Mean	6.94	8.33					
Butyrate	200	4.14	4.50	4.32	0.097	0.2285	0.1944	0.5461
	400	4.52	4.64	4.58				
	Mean	4.33	4.57					
Isobutyrate	200	0.18	0.20	0.19	0.006	0.1187	0.1709	0.5857
	400	0.20	0.21	0.20				
	Mean	0.19	0.21					
Valeriate	200	0.32	0.35	0.33	0.008	0.0016	0.0108	0.3775
	400	0.34	0.38	0.36				
	Mean	0.33	0.36					
Isovaleriate	200	0.26	0.29	0.27	0.008	0.7606	0.3682	0.2967
	400	0.30	0.28	0.29				
	Mean	0.28	0.28					
Capronate	200	0.11	0.13	0.12	0.009	0.0307	0.0565	0.3714
	400	0.12	0.17	0.15				
	Mean	0.11	0.15					
Acetate / Propionate ratio	200	4.21[a]	3.98[a]	4.10	0.081	< 0.001	0.0067	0.0297
	400	4.14[a]	3.50[b]	3.82				
	Mean	4.18	3.74					

Values represent means with SEM (n=5).
[1]MWP untreated with ozone lamp was used as a substrate for ruminal fermentation.
[a-c]Mean values within a short chain fatty acid or a ratio of acetate to propionate not sharing a common superscript letter were significantly different, *P*< 0.05.

Table 4. Effects of sterilization lamp- and ozone lamp-irradiated wood powder (MWP) on *in vitro* ruminal fermentation for 48 hours (Experiment 4).

	MWP (mg)	UV-light irradiation			Mean	SEM	ANOVA (P-value)		
		Control[1]	Sterilization lamp	Ozone lamp			Treatment	Amounts	Interaction
		Changes in concentrations (mmol/l)							
Total volatile fatty acids[2]	200	1.66	3.86	5.07	3.53	0.693	0.0211	0.0038	0.86671
	400	4.58	7.39	9.46	7.14				
	Mean	3.12[b]	5.63[ab]	7.27[a]					
Acetate	200	1.32	2.06	3.22	2.20	0.417	0.0398	0.0106	0.82997
	400	2.68	4.31	5.55	4.18				
	Mean	2.00[b]	3.18[ab]	4.38[a]					
Propionate	200	0.58	1.52	1.63	1.24	0.210	< 0.001	< 0.001	0.28903
	400	1.56	2.54	3.53	2.54				
	Mean	1.07[b]	2.03[ab]	2.58[a]					
Butyrate	200	-0.12	0.35	0.19	0.14	0.103	0.5550	0.3117	0.78486
	400	0.30	0.40	0.40	0.36				
	Mean	0.09	0.37	0.29					

Values represent the changed concentrations from their values in blank samples incubated the only mixed rumen fluid without substrates, and   means with SEM (n=5 animals).
[1]MWP untreated with UV lamps was used as a substrate for ruminal fermentation.
[2]Total volatile fatty acid concentrations include major short chain fatty acids of acetate, propionate and butyrate, and minor short chain fatty acids of isobutyrate, valeriate, isovaleriate, capronate.
[a,b]Mean values within a short chain fatty acid not sharing a common superscript letter were significantly different, *P*< 0.05.

consistent with our companion article [17]. However, the capacity for volatile fatty acid production still remained small, and a particle size of micronised wood powder less than 100 μm was not an important factor affecting its ruminal fermentation.

Plant cell wall digestion is dependent on rumen microorganisms consisting of bacteria, protozoa, and fungi. It has been known that ruminal cellulolytic activity is affected by different species of microorganisms [28,29]. In *in vitro* monoculture, Lee *et al.* [28] reported that plant cell walls were more digestible by fungi than by bacteria. Moreover, this activity will be inhibited by development

of cross-linkages of lignin [9]. The present results suggested that ruminal fermentation of micronised wood powder was dependent on the interrelationship between cellulolytic activity of rumen microorganisms and the presence of lignin, even though the particle size of micronised wood powder was reduced to increase the degree of exposure of holocelluloses.

In Experiment 2, the methanol treatment of micronised wood powder apparently improved volatile fatty acid production dose-dependently. Because methanol is known to dissolve and partially remove lignin from lignocellulose [23], the cellulolytic activity of rumen microorganisms on micronised wood powder might be enhanced by reducing the lignin concentration in the wood powder. Furthermore, this study found that propionate production was sensitive to fermentation of methanol-treated micronised wood powder, resulting in a reduced ratio of acetate to propionate. Although the mechanism that led to the increased propionate production by the methanol-treated micronised wood powder remained unclear, the altered volatile fatty acid composition is interesting as a characteristic response. If lignin is removed from micronised wood powder, the cellulolytic activity of rumen microorganisms may be elicited to stimulate propionate production.

Micronised wood powder treated with methanol stimulated volatile fatty acid production. In fact, the use of a solvent to remove woody substances such as lignin but preserve holocelluloses will additionally increase the labor and cost of micronised wood powder production due to the processes of filtering, washing, and drying the methanol-treated micronised wood powder. In Experiment 3, micronised wood powder was irradiated with an ozone lamp to examine the decomposition of lignin. It has been reported that exposure of lignocellulose to ozone decomposed it to lignin [20,21,30]. Because the mixing for preparation to expose micronised wood powder to a sterilisation lamp (254 nm) and ozone gas was performed manually, decomposition of lignin might have been insufficient in this study. In fact, whether ozone-lamp irradiation induced oxidative decomposition of lignin in micronised wood powder was unclear because this study did not determine the chemical alteration of lignin.

Consequently, the preparation with the ozone lamp was effective for volatile fatty acid production by micronised wood powder fermentation (Experiment 3). The chemical change in lignin might be responsible for stimulated cellulolytic activity of rumen microorganisms. Furthermore, this study confirmed that irradiation of micronised wood powder by an ozone lamp was superior to that of a UV lamp (254 nm) for accelerating volatile fatty acid production (Experiment 4). In the combination of both UV wavelengths, 254 nm UV would play a role to stimulate generation of active oxygen (free radicals) by ozonolysis [30]. Thus, an ozone lamp consisting of UV wavelengths of 254 nm and 185 nm will be effective to elicit the photochemical decomposition of lignin. However, Cogulet *et al.* [18] reported that irradiation of stems of white spruce at 340 nm UV-A is able to degrade lignin. If a combination of different UV-A wavelengths is used for pretreatment of micronised wood powder, the cellulolytic activity of rumen microorganisms may be accelerated in ruminal fermentation of micronised wood powder.

Overall, in volatile fatty acid production, the major fermentation products induced by micronised wood powder were acetate and propionate. Additionally, micronised wood powder treated with an ozone lamp stimulated propionate production to the same extent as the methanol-treated micronised wood powder. This characteristic may affect subsequent *in vivo* metabolic utilisation of volatile fatty

acids in the body. On the contrary, El-Sabban *et al.* [3] suggested that fine sawdust passed quickly through the gastrointestinal tract in heifers. Likewise, we reported that feeding untreated micronised wood powder had no effect on the total volatile fatty acid concentration in rumen fluid obtained from beef cattle [17]. It remains uncertain whether treatment of micronised wood powder with an ozone lamp induces rapid fermentation for volatile fatty acid production in *in vivo* rumen fluid. Further *in vivo* experimentation is needed to elucidate the fermentation kinetics of micronised wood powder treated with an ozone lamp.

On the other hand, the acceptability of micronised wood powder produced from Japanese cedar is markedly low in relation to its aromatic woody odor. In this respect, irradiation of UV light using both a sterilisation lamp (254 nm) and an ozone lamp completely removed the unique wood aroma of Japanese cedar. This may improve cattle acceptance of micronised wood powder.

In conclusion, *in vitro* ruminal fermentation of lignocellulose in micronised wood powder was not associated entirely with its particle size. This study found that ruminal fermentation of micronised wood powder was improved merely by removing methanol-soluble substances from the wood powder. Moreover, in a pretreatment that did not require wetting the wood powder, irradiation of micronised wood powder with an ozone lamp was effective to enhance the fermentation contributing to volatile fatty acid production. Therefore, the present study suggested the possibility that micronised wood powder from lignified biomass is useful as a feedstuff, being an energy source capable of volatile fatty acid production directly by ruminants.

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