Effect of Mixed Microbial Culture on Fermentation of Beverage Residues and the Effect of the Fermented Beverage Residues on in Vitro Rumen Fermentation and Methane Production

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***Abstract*—The aim of this study was to increase the nutritive value of coffee, green and oolong tea residues and assess if the fermented residues have the potential to mitigate enteric methane emissions. A mixed microbial culture (20 g/kg) was added to each residue and the mixture incubated for 3 d at 35 ℃ (anaerobically) and 2 weeks at 30 ℃ (aerobically). Unfermented and fermented beverage residues were assayed for their saccharide and ethanol content. Four separate total mixed rations (TMR) were prepared using the three fermented beverage residues and hay (control). The fermented residues (2 g dry matter (DM)) were individually mixed with hay (3 g DM) and concentrate (5 g DM) and the in vitro rumen methane output was quantified during the 24 h incubation period using the continuous gas quantification system. The fermented residues of coffee, oolong tea and green tea had higher concentrations of cellotriose, cellobiose and xylobiose than the corresponding unfermented residues. Ethanol concentration was higher in the fermented coffee and green tea residues than in their respective unfermented residues. The methane output (L CH4/24 h) from the TMR containing fermented residues of coffee (0.118 L), oolong tea (0.127 L) and hay (control; 0.123 L) did not differ, but the methane output was lower for all compared to the TMR containing fermented green tea residue (0.141 L). The results suggest that fermented coffee, oolong tea and green tea residues are a potentially good source of protein and energy, and fermented residues of coffee caused a numerical decrease methane output.**

***Index Terms*—Beverage residues, in vitro, methane, mixed microbial culture.**

I. Introduction

Over the last decade, livestock farmers have faced the problem of acquiring affordable feed for their livestock. Hence, agricultural and industrial by-products have been suggested as one of the solutions. However, this feed source is usually of low quality and the some of these by-products may have a negative effect on animal production [1], because of their low nitrogen and high fiber content. The high fiber content prevents assess of ruminal hydrolytic enzymes to cellulose and hemicellulose [2], [3]. During recent years, mixed microbial culture and their fibrolytic enzymes have been used to improve the nutritive value and utilization efficiency of low-quality roughages. Agricultural by–products are produced in large quantities throughout the world and could potentially consist of nutrients to form part of the ruminant diet. With coffee and tea being some of the most common beverages consumed daily around the world, the safe disposal of large quantities of unused residues in the producing countries is challenging and the release of these residues into the environment is causing enormous problems. In addition, methane output from beverage residues mixed total ration on in vitro fermentation represents a loss of energy to the host animal [4], and [5] contributes to global greenhouse gas emissions [6]. Therefore, the objectives of this study were to evaluate the effect of a mixed microbial culture treatment on the nutritive value of coffee, green tea and oolong tea beverage residues and the effect of the fermented residues on in vitro rumen fermentation and methane production.

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II. Materials and Methods

A mixed microbial culture (20 g/kg dry matter (DM); Bio-PKC®, Marubeni Corporation, Tokyo, Japan) was mixed separately with 1 kg of coffee, green tea and oolong tea residues, placed into sealable bags and incubated for 3 d at 35 ℃ (anaerobically) and 2 weeks at 30 ℃ (aerobically).

A. Sample Preparation and in Vitro Fermentation

The fermented beverage residues were oven-dried at 60 ℃ for 48 h and stored under dry, cool, dark conditions in sealed containers prior to use. Four total mixed rations (TMR) were prepared that contained either a beverage residue or hay (200 g/kg DM) and hay (300 g/kg DM) and concentrate (500 g/kg DM). The TMR treatments evaluated were: hay (Control-TMR), coffee residue (C-TMR), green tea residue (G-TMR), and oolong tea residue (O-TMR). The effects of each TMR treatment (10 g DM) on rumen fermentation were tested in vitro for 24 h at 39 ℃ using the continuous gas quantification system as previously described by [7]. Briefly, samples of rumen fluid were obtained from two non-lactating Holstein cows prior to morning feeding, strained and combined on an equal volume basis. Buffer was prepared according to reference [8], sterilized by autoclaving and flushed with CO_2 for 1 h prior to being dispensed into each fermentation jar. Rumen fluid was added to buffer at a ratio of 1:4. Samples of the incubation medium were collected at intervals of 0, 2, 4, 8 and 24 h and stored at -20 ℃ for

subsequent ammonia-N (NH_3-N) and volatile fatty acid (VFA) analysis. At the end of each 24 h incubation period, all incubations were stopped, the contents were discharged, and the fermenters were thoroughly washed and autoclaved. The fermenters were then re-charged with fresh buffer and inoculum to begin the next 24 h incubation period. The experiment was repeated four times in total with treatments randomly assigned to incubation jars for each incubation period.

B. Volatile Fatty Acids and Methane Analysis

Volatile fatty acid concentrations were determined by gas chromatography (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (ULBON HR-52, 0.53 mm ID ×30 m, 3.0 μm) using 2-ethyl-n-butyric acid as an internal standard and samples were prepared for analysis according to [7]. Methane output from each fermentation jar was continuously measured by infrared analyzer (Ex air, Yokogawa, Tokyo, Japan) using the in vitro continuous gas quantification system (Takasugi Seisakusho, Tokyo, Japan) and all data were pooled to a computer interface at 1 min intervals. The pH and oxidation reduction potential (ORP) were measured using a pH and ORP meter (HM-21P, Toa electronics Ltd., Tokyo, Japan).

C. Chemical Analysis

Unfermented and fermented beverage residues were dried at 60 ℃ for 48 h in a forced-air oven and then ground through a 1 mm sieve. Samples were stored at room temperature in sealed containers. Duplicate samples of each residue were assayed for DM, crude protein (CP), organic matter (OM), neutral detergent fibre (NDF) and acid detergent fibre (ADF). Dry matter and OM of the samples were determined according to the AOAC procedures [9]. Nitrogen (*N*) was determined by the Kjeldahl method [9] using an electrical heating digester (FOSS tecator™ Digester, Tokyo, Japan) and an automatic distillation apparatus (FOSS kjeltec™ 2100, Tokyo, Japan), and then CP was determined as $N \times 6.25$. Neutral detergent fibre and ADF were determined according to the methods as in [10]. The concentrations of individual saccharides were determined by high performance liquid chromatography (HPLC) as described by [11]. Ammonia-N concentration in the incubation medium was analyzed according to the procedure of [12].

D. Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) using PROC GLM in SAS statistical software version 9.2 [13]. Differences among means were identified using Tukey multiple comparisons. Effects were considered significant when $P<0.05$.

III. Results and Discussion

A. Saccharides and Ethanol Concentration of Unfermented and Fermented Beverage Residues

TABLE I: Saccharides and Ethanol Concentration (Mg/G Organic Matter) of Unfermented and Fermented Beverage Residues.

Type	Treatment	Variables						
		Cell	Cellb	Xylb	Xyl	Arab	Glu	Ethnl
Coffee	Unfermented	0.03	0.01	0.01	0.03	0.01	ND	0.01
	Fermented	5.06	2.20	0.99	1.34	2.52	ND	1.89
	S.E.M.	0.038	0.038	0.011	0.002	0.823	-	0.021
	P	0.01	0.02	0.01	0.21	0.38	-	0.01
Green tea	Unfermented	1.15	0.14	0.54	0.14	0.33	0.10	0.03
	Fermented	3.81	4.36	11.87	9.09	1.90	5.13	8.50
	S.E.M.	0.070	0.049	0.106	0.254	0.251	0.328	0.777
	P	0.01	0.01	0.04	0.21	0.09	0.01	0.02
Oolong tea	Unfermented	0.01	0.07	0.02	0.01	0.01	0.06	ND
	Fermented	5.42	4.75	3.33	1.88	1.66	3.81	1.09
	S.E.M.	0.116	0.042	0.074	0.060	0.055	0.024	0.770
	P	0.03	0.01	0.03	0.04	0.04	0.009	0.57

Cell, cellotriose; Cellb, cellobiose; Xylb, xylobiose; Xyl, xylose; Arab, arabinose; Glu, glucose; Ethnl, ethanol; ND, not detected; S.E.M, standard error of the mean.

The saccharides and ethanol concentration of unfermented and fermented beverage residues are presented in Table I. The fermented residues of coffee, oolong tea and green tea had higher ($P<0.05$) concentrations of cellotriose, cellobiose and xylobiose than the unfermented residue of each beverage. In addition, fermented oolong tea residues were higher in arabinose ($P=0.04$), glucose ($P=0.009$) and xylose ($P=0.04$) compared to its unfermented residue. The high concentration of saccharides may be associated with ligno-cellulolytic enzymes produced by microbes during anaerobic and aerobic fermentation [14]. There was higher ethanol concentration in the fermented coffee, green and oolong tea residues than their unfermented residues. Higher ethanol production might have been stimulated by yeasts contained in the MMC during aerobic fermentation. It has been reported that greater ethanol production can be seen during aerobic fermentation when sufficient amount of sugars are available for yeast to maintain their metabolism [15]-[17].

A. Chemical Composition of Unfermented and Fermented Beverage Residues

The chemical compositions of unfermented and fermented beverage residues are presented in Table II. The CP concentration did not differ ($P>0.05$) for any of the beverage

residues after MMC fermentation. However, the CP concentration in both the unfermented (range, 131.7 – 304.1 g/kg DM) and fermented (range, 140.0 – 312.6 g/kg DM) residues was high, and therefore these residues are a potentially good source of protein for livestock. Coffee and tea residues can contain tannins, which might explain the lack of an increase in CP due to MMC fermentation. In addition, the maillard reaction and heat damage could occur during beverage production under high heat. Referece [18], and [19] have indicated these reactions and damages that are highly resistant to microbial enzyme to cell wall degradation. Hence, this might be another reason for a lack of an increase in CP in fermented residues. Neutral detergent fibre was reduced in fermented green (P=0.01) and oolong tea (P=0.05) residues compared to their unfermented residues. This result agrees with the findings of [20] and [21] who suggested that the reduction in NDF could be due to the stabilization characteristics of the microbial inoculant. Acid detergent fibre concentration was lower (P=0.006) for the fermented than unfermented coffee residue.

B. *In Vitro Rumen Fermentation Characteristics*

In vitro rumen methane output, carbon dioxide output (CO_2), NH_3-N concentration, pH, ORP and VFA production after 24 h incubation are displayed in Table III. The methane output (L CH_4/24 *h*) from C-TMR (0.118 *L*), O-TMR (0.127 *L*), and Control-TMR (0.123 *L*) did not differ from one another, but the methane output was lower for all compared to G-TMR (0.141 *L*). The increase in CH_4 output observed for G-TMR may reflect the increase in DM of the fermented residue. It is known that higher fibre concentrations in a diet can increase the CH_4 output [22]. There was a numerically lower CH_4 output with C-TMR compared to the other TMR treatments. Coffee residues contain considerable amounts of phenolic compounds such as tannins, saponins and caffeine [1], [23]-[26]. Reference [25] observed that tannin-containing forage diets have the potential to reduce CH_4 emissions in ruminants. In addition, saponins can improve rumen fermentation and nitrogen metabolism [27]. Furthermore, saponins may inhibit rumen methanogen populations through a reduction in protozoa numbers [28], because methanogens have both ecto- and endo-symbiotic relationships with protozoa [29]. Consequently, methanogen species associated with protozoa usually decrease with decreasing protozoa numbers [30]. Methanogens associated with protozoa may account for decreased CH_4 emission [31].

TABLE II: DRY MATTER (DM, G/KG), CRUDE PROTEIN (CP), ORGANIC MATTER (OM), NEUTRAL DETERGENT FIBRE (NDF) AND ACID DETERGENT FIBRE (ADF) (G/KG DM) COMPOSITION OF UNFERMENTED AND FERMENTED BEVERAGE RESIDUES.

Type	Treatment	Variables				
		DM	CP	OM	NDF	ADF
Coffee	Unfermented	956.5	131.7	983.2	333.1	458.9
	Fermented	982.5	140.0	988.1	332.5	353.4
	S.E.M.	0.10	0.17	0.03	0.03	0.04
	P	0.05	0.25	0.09	0.44	0.006
Green tea	Unfermented	952.0	304.1	962.7	411.4	336.6
	Fermented	963.4	312.6	958.7	550.3	245.0
	S.E.M.	0.01	0.90	0.20	0.10	0.47
	P	0.02	0.54	0.49	0.01	0.07
Oolong tea	Unfermented	921.5	208.7	957.0	548.8	298.3
	Fermented	965.6	214.0	956.3	465.5	294.6
	S.E.M.	0.23	0.79	0.06	0.31	0.25
	P	0.07	0.84	0.55	0.05	0.53

S.E.M, standard error of the mean.

TABLE III: IN VITRO METHANE (CH4) OUTPUT, AMMONIA-N (NH3-N) CONCENTRATION, PH AND VOLATILE FATTY ACID (VFA) PRODUCTION FROM TOTAL MIXED RATIONS (TMR) AFTER 24 H INCUBATION.

	Control TMR	Coffee TMR	Green tea TMR	Oolong tea TMR	S.E.M.	P
CH_4 (L/24 h)[1]	0.123[b]	0.118[b]	0.141[a]	0.127[b]	0.003	0.009
CO_2 (L/24 h)[1]	1.57[b]	1.41[b]	2.41[a]	1.47[a]	0.222	0.008
NH_3-N (mg/L)[1]	37.44	39.22	35.77	29.13	3.311	0.528
pH[2]	6.86	6.84	6.69	6.82	0.066	0.600
ORP[2] (mV)	-399.75	-394.50	-418.75	-393.75	10.52	0.706
Total VFA (mM)[1]	37.95	39.43	48.30	37.49	4.431	0.307
VFA (mM)[1]						
Acetic (A)	26.69	27.43	33.50	27.07	3.526	0.422
Propionic (P)	10.54	11.38	14.03	9.84	1.033	0.086
Butyric	0.714[ab]	0.623[ab]	0.770[a]	0.573[b]	0.040	0.048
A:P ratio	2.49	2.42	2.51	2.64	0.214	0.493

[1]Mean values after 24 h incubation (n=4). [2]Mean values over the 24 h incubation period (n=4).
[a-b]means within a row with different superscripts are significantly differ (P<0.05).

The carbon dioxide output (L CO_2/24 h) from C-TMR (1.41 *L*), O-TMR (1.47 *L*), and Control-TMR (1.57 *L*) were not significantly different, but the CO_2 output of G-TMR (2.41 *L*) was significantly different. The increase in CO_2 output may reflect the increase the CP content of the fermented G-TMR. In addition CO_2 is an end product of

lactate fermentation to propionate via the succinate fermentation partway by the ruminal bacterium [32]. Therefore the increase CO_2 production might promote growth and activities of rumen microorganisms in the fermentation jars. Concentrations of ammonia-N, total VFA (tVFA), acetic acid (A), and propionic acid (P), and pH did not differ between TMR treatments, but the G-TMR had a numerically higher concentration of tVFA than the other TMR treatments. The addition of agricultural by-products to the diet of livestock may enhance the VFA concentration [33]. Reference [34] reported that, roughage based diets might increase the tVFA and decrease the A:P ratio. Both reference [25], and [35] reported that tVFA varies with the response to phenolic compounds in the diet by depressing fibre degradation, with the extent of which depends upon the chemical structure of the phenolic compound and the species of methanogen [36]. Although not observed in this study, reference [7] reported that an increasing pH might decrease tVFA. In a study to determine the fermentation quality of TMR silage containing coffee residues, butyric acid was not detected because of the inhibition of its production by coffee residues [37]. Whereas, in this study, butyric acid was detected and concentrations differed across treatments (P=0.048). Butyric acid concentration was higher (P=0.048) in G-TMR than O-TMR and neither of those two residues differed to the Control-TMR and C-TMR. Oxidation reduction potential can change slightly with the substrate type [38] and it is an indicate measure of the microbial activity. Rumen is anaerobic condition with potentially negative ORP [39], [40]. In this study, ORP did not change between TMR treatments but G-TMR had a numerically lower value.

I. Conclusion

Beverage by−products such as coffee, oolong tea, and green tea residues are a good potential source of energy and protein. Moreover, the methane output of C-TMR was numerically lower than the other TMR treatments. By fermenting coffee, oolong tea and green tea by-products with a mixed microbial culture, these beverage residues have the potential to help eliminate some of environmental pollution problems that exist today in coffee and tea producing countries, as well as provide an affordable feed for livestock. However, the current use of beverage by-products is limited due to their high phenolic acid content. Therefore, further research is needed to determine the dry matter intake, the health implications of feeding beverage residues, the appropriate method of offering the feedstuffs and the optimum levels of incorporation into diets.

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