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Comparing Tea Leaf Products and Other Forages for *In-vitro* Degradability, Fermentation, and Methane for Their Potential Use as Natural Additives for Ruminants

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Abstract

Tea leaves are a rich source of plant secondary metabolites such as tannins and saponins that have the potential to manipulate rumen fermentation and to lessen methane (CH_4) production.Samples of green tea (GTL), black tea (BTL), their spent leaves after water extraction (SGTL and SBTL), ryegrass hay (RH), ryegrass silage (RS), paddy straws (PS), barley straws (BS), and wheat straws (WS) were compared for their rumen *in-vitro* organic matter degradability (IVOMD, g/kg DM), pH, ammonia (NH_3, mg/L), total volatile fatty acids (tVFA, mmol/L), total gas production (tGP, L/kg OM), and methane output (CH_4,L/kg OM) after 28h incubation with buffered rumen fluid under anaerobic conditions at 39°C in glass syringes. One-way ANOVA on Minitab 16 was used to examine differences between products at $P<0.05$ for four replicate samples. There were no differences between tea leaf products, RH and RS but the straws tended to have lower IVOMD compared with tea leaf products and other forages. GTL produced the lowest NH_3 followed by BTL, SGTL, SBTL, and other forages. There were no differences between most tea leaf products, RH, RS, and the straws for tVFA concentration but PS and WS produced the lowest tVFA. GTL, SGTL, and RH had higher tGP than BTL, SBTL, and the straws but they had a lower tGP than RS. GTL, BTL, and SBTL produced similar levels of CH_4 as the straws but this was less than RS and SGTL. The results suggest that if tea leaf products are included in the straw-based diets as natural feed additives, they may improve degradability, tVFA, NH_3,and tGP production without increasing CH_4output. Low NH_3 production for tea leaf products could be the sign of more by-pass protein to be absorbed in small intestine.

Keywords: Tea leafproducts, in-vitro measurements, and ruminants.

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1. Introduction

Teas and their spent tea leaves (STL) as residues are potentially good sources of protein, minerals, and plant secondary metabolites [1]. In ruminants, plant secondary metabolites such as phenols and tannins may increase the availability of rumen bypass protein and non-ammonia nitrogen (NH_3 N) supply which can be absorbed in the small intestine due to their binding ability to plant proteins [2–5]. Although NH_3 is an important source of N for rumen microbes, its over or fast production may exceed the ability of microbes to utilize it. This can lead to an excessive NH_3 supply that after absorption through rumen wall can enter the blood stream, liver, and eventually excreted in urine as an N waste [6, 7]. Tannins have the potential to reduce rumen CH_4 production [3, 5]. Similarly, tea saponins can reduce CH_4 and NH_3 productions [8-10] by reducing protozoa and the methanogenic activity of relevant microbes [8, 9].CH_4 and NH_3 are energetically wasteful end products of rumen fermentation so that the reduction in CH_4 production in the rumen is assumed to be the reflection of more efficient feed utilization [8]. Agricultural activities are supposed to be responsible for 40-60% of the total anthropogenic CH_4 production while 25-40% of this comes from livestock sector, predominantly from ruminants through their eructation and manures [11-13]. CH_4 production is also associated with the loss of gross energy by 2-12% [14]. Hence, CH_4 mitigation in ruminants is an aim, not only for environmental advantage, but also for feed utilization efficiency. Lastly, if the chemical properties of tea products are able to manipulate rumen fermentation, these products can be used as a natural alternative to replace growth-promoting antibiotics that have been banned in the European Union since 2003 (1831/2003; EC, 2003) and which may also be banned in other countries such Asian Australasian in the future. Therefore, this study aimed to compare tea leaf products and other feeds for *in-vitro* degradability, fermentation, and CH_4 production for their potential use to manipulate rumen fermentation.

2. Material and Methods

2.1. Sample preparation

Green (GTL) and black (BTL) tea leaves were obtained from a tea processing company (PT. Kabepe Chakra), located in Bandung, West Java, Indonesia. GTL was graded as *Sow Mee* (Code: SM #315) and the black tea was graded as *Broken Orange Pekoe Fanning* (Code: BOPF #355). STL were obtained by extracting 2.8 g of either green (SGTL) or black (SBTL) tea leaves in a fixed volume of 300 ml of boiling water for 5 minutes. While samples of ryegrass hay (RH), ryegrass silage (RS), barley straws (BS), and wheat straws (WS) were collected from Cockle park farm, Newcastle University UK

during April 2012. The sample of paddy straws (PS, variety IR50) was obtained from Bangladesh in a dried form. Before chemical analysis, all samples were oven dried at 60°C and ground through 1 mm sieve using a sample mill.

2.2. Chemical analysis

The AOAC methods [15] were used to determine dry matter (DM), ash, organic matter (OM) and ether extract (EE) while total nitrogen (N) (N × 6.25 = Crude Protein, CP) was analyzed by Elementar Vario Macro Cube (Elementar, Hanau Germany). The neutral detergent fibre (NDF) content was determined according to Van Soest [16] but without using *amylase*, sodium sulphite, and dekalin while acid detergent fibre (ADF) was determined as reported by Van Soest [17]. The NDF and ADF contents were calculated by excluding ash. Metabolisable energy (ME) was calculated by using the formula of Khan and Chaudhry [18].

2.3. Buffered inoculum

The rumen fluid (RF) for this *in-vitro* experiment was collected on 5 June 2013 from two freshly slaughtered grass-fed lambs (Texel Cross) from a local slaughterhouse located at Buradon, Newcastle upon Tyne UK.Immediately after slaughtering, the rumen was cut and RF was directly filtered through two layers of muslin cloth on a large funnel connected to pre-warm insulated thermos flasks (Thermos Ltd, UK) until fully filled and closed tightly allowing anaerobic conditions to be maintained inside the flasks, and then transported directly to the Laboratory for immediate use within 1 hour of collection.After thatRF was mixed, quantified, and transferred quickly, under filtration oftwo layers of muslin cloth, into the pre-warmed dark bottles (2.5 L capacity) containing buffer solution [19] at 1:2 ratio (RF:buffer) while kept in a water-bath (39°C). The bottles containing buffered RF were then purged with CO_2 to remove oxygen and tightly closed with a dispenser (50 ml capacity, Fisher Scientific UK). The pH of each buffered inoculum was adjusted around 7 ± 0.2 using HCL drop wise.

2.4. *In-vitro* incubation

About 200 ± 4 mg of each sample was transferred into a 50 ml glass syringe (SAMCO, UK), lubricated with Vaseline, and fitted with a 4 way-male-slip stopcock (Cole Palmer Instrument, UK). About 20 ml buffered inoculums was added to each syringe which was closed and placed in a shaking water bath at 39°C. Total gas production (tGP) in each syringe was measured every two hours for up 28h.After incubation, most of the warm water in the water bath was replaced with sufficient ice to stop further

fermentation in the syringes. About 15 ml gas from each incubated syringe was then transferred into another clean syringe from where the gas was transferred to a 12 ml evacuated gas tube (Labco Exetainer, Labco Ltd, UK) by using a needle being attached to the stopcock for CH_4 analysis. Furthermore, all the contents in each syringe (inoculum and the residues) were transferred into a pre-weighted tube (polyethylene, 50 ml capacity) for the pH, NH_3, VFA, and *in-vitro* organic matter digestibility (IVOMD) measurements. pH was measured directly by a pH meter (pH 309, Hanna Instruments Ltd, UK). All tubes were then centrifuged and subjected to sample preparations for volatile fatty acids (VFA) and NH_3 analyses. All the remaining residual particles in the syringes were water washed into the corresponding tubes containing the residues. These undigested residues were dried at 80°C for IVOMD determination as described by Khan and Chaudhry [17]. Two blank representatives were run alongside the samples in each trial and the blank values were used to correct the degradability and tGP estimations.

2.5. VFA and NH_3 analyses

After centrifugation, 2 ml of each supernatant was pipetted into a capped-container and mixed with 0.5 ml of deproteinising solution containing 200g/L metaphosphoric acid and 10 mmol/L of crotonic acid as an internal standard for VFA determination. About 2 ml of each sample was then transferred into 2 ml gas chromatograph (GC) vial ready for analysis along with a mixed VFA standard. A mixed standard solution contained (mmol/L) acetate (50), propionate (20), iso-butyrate (10), n-butyrate (10), iso-valerate (10), n-valerate (10), and crotonic acid (10). All individual VFA standards were purchased from Sigma-Aldrich, UK. A set of GC, Shimadzu GC-2014 (Kyoto, Japan) with a capillary GC column (15m × 0.53 mm × 1.20 μm film thickness) (Econo-Cap EC-1000, Altech, UK) and an auto injector (Shimadzu, AOC-20i) was connected to Shimadzu GC solution software which controlled almost all the operations of this VFA analysis. Purified helium was utilized as a carrier gas with a head pressure of approximately 3.4 kPa and a column flow of 0.85 ml/min. Peaks were detected by flame ionization detection (FID). A split injection system on an auto sampler was used with a split ratio of 34.5:1 and an injector temperature of 250°C while the detector temperature was 275°C. A 1μl sample injection was applied when the initial temperature of the column was at 120°C. It was then raised at 10°C/minute to 240°C in 12 min. Furthermore, the temperature was then decreased at 60°C/min back to 120°C in 2 min to give a final gradient with the total runtime of 17 minutes. The data, including peak areas and chromatograms were extracted from Shimadzu GC solution software after the analysis. Total VFA (tVFA) was calculated as the sum of acetate, propionate, iso-butyrate, n-butyrate, iso-valerate, and n-valerate. Meanwhile, NH_3 determination was prepared

by pipetting 2 ml of each supernatant into a capped-container and acidifying them with 2 ml of 1 (N) HCl. NH_3 was analysed by Pentra 400 (Horriba Ltd, Kyoto, Japan) with calibrated standards of NH_3-N at 25, 50, and 100 μg/ml in pure distilled water.

2.6. CH_4 determination

CH_4 determination was performed using a GC-MS (Fisons 8060 GC, Italy) using split injection (150°C) linked to a Fisons MD 800 MS (electron voltage 70 eV, emission current 150 μA, source current 600 μA, source temperature 200°C, multiplier voltage 300V, interface temperature 150°C). The acquisition was controlled by a Compaq Deskpro computer using Xcalibur software (Xcalibur Inc. USA) in a full scan mode (1.0-151.0 amu/sec). A headspace gas sample of 100μl using a 100μl GC syringe (SGE Europe Ltd, UK) was injected in duplicate in a split mode into the HP-PLOT-Q capillary column (30m × 0,32mm i.d) packed with 20μm Q phase (J&W Scientific, USA) of the GC. The GC was held isothermally at 35°C with Helium as the carrier gas (flow 1 ml/min, pressure of 65kPa and open split at 120 ml/min). The chromatograms of the separated gase (CH_4) were integrated and quantified. A calibrated mixture gas of 60% CH_4in CO_2(Scientific & Technical gases Ltd, UK) was run along with the samples at 20, 40, 60, 80, and 100 μl injections to suit the standard curve calibrations.

2.7. Statistical analysis

Chemical compositions of tea leaf products and forages were averagely calculated from triplicate analysis while one-way analysis of variance (ANOVA) on Minitab 16 software was used to compare different tea leaf products and other forages (4 replicates each) for their *in-vitro* degradability, fermentation, and CH4 output. Differences were considered significant if $P < 0.05$.

3. Results and Discussion

Chemical compositions (g/kg DM) of various tea leaf products and other feed types were described in Table 1. Tea leaf products had greater CP than RH, RS, and the straws. Tea leaf products had also higher ME but lower Fibre fractions than the straws. Here, the straws had the lowest CP and ME but highest in Fibre fractions compared with tea leaf product and other forages. In addition, GTL and BTL had less fibre fractions, higher ash but almost the same EE and ME contents than their corresponding STL.

There were no differences between tea leaf products, RH, and RS on IVOMD but the straws tended to have lower IVOMD than tea leaf products and other forages. There were no differences between tea leaf products and most of forages on rumen

Table 1: Chemical compositions of tea leaf products and other forages (g/kg DM).

Forages	DM	OM	Ash	CP	EE	NDF	ADF	ADL	ME
GTL	937	938	61.8	240	20.8	254	211	37.6	7.08
BTL	942	939	61.4	242	12.6	323	309	27.4	6.40
SGTL	134	957	43.3	246	23.1	405	294	40.3	7.39
SBTL	126	961	38.7	234	13.5	474	410	44.5	6.59
RH	840	908	92.4	200	20.2	649	507	435	6.79
RS	325	917	83.4	136	14.0	595	427	379	7.60
PS	944	818	182	60.4	9.9	787	684	598	4.01
BS	866	948	51.6	49.1	18.1	846	672	594	4.34
WS	903	938	62.5	38.1	46.1	843	590	530	4.43

[1]ME (MJ/ kg DM) was calculated by the formula of Menke and Steingass (1988); GTL, green tea leaves; BTL, black tea leaves; SGTL and SBTL, green and black spent tea leaves; RH, ryegrass hay; RS, ryegrass silage; PS, paddy straws; BS, barley straws; WS, wheat straws.

pH except GTL had lower pH than the straws. GTL produced the lowest NH_3 followed by BTL, SGTL, SBTL, and other forages. There were no differences between most tea leaf products, RH, RS, and the straws for tVFA concentration but PS and WS produced the lowest tVFA. GTL, SGTL, and RH had higher tGP than BTL, SBTL, and the straws but they had lower tGP than RS. GTL, BTL, and SBTL produced similar levels of CH_4 as the straws but this was less than RS and SGTL.

Based on this *in-vitro* experiment, the mean IVOMD of tea leaf products was higher than the straws but slightly lower than the RH and RS. The tea leaf products were degraded more in the rumen than the straws. The rate of tea leaf product degradation was close to those of higher quality forages such as RH and RS. This higher degradability was in line with the higher CP and ME but lower fibre contents in the tea leaf products alongside RH and RS compared with the straws. This observation also confirmed that GTL were more degradable in the rumen than BTL counterpart which might have acquired more resistant components due to the 'Maillard browning reactions' during black tea manufacturing. As expected, the higher nutritive values and IVOMD of tea leaf products than straws resulted in a significant greater tGP of tea leaf products in comparison with the straws. It was good to observevariable tGP as its volume changed with the change in the substrate type. In fact, tGP has been reported to be positively correlated with ME content in the diet and ME was positively correlated with the CP and EE contents [16, 19]. Meanwhile, lower NH_3 for most tea leaf products than other forages could be attributed to their high tannin contents [1]that have the ability to modify the microbial activity in the rumen. Tannins can bind and protect plant proteins from rumen digestion and thus reduce NH_3 production [2–5].

Although original tea leaves such as GTL and BTL had higher rumen IVOMD and tGP than the straws, their CH_4 outputs as L/kg OM, were not different. This means that the concentration of CH_4in the total gas for GTL and BTL were significantly lower than that

Table 2: Means (± SD) for rumen IVOMD (g/kg DM), pH, tVFA (mmol/L), NH_3 (mg/L), tGP (L/kg OM), and CH_4 (L/kg OM) of tea leaf products and other forages at 28h of incubation.

Forages	IVOMD	pH	tVFA	NH_3	tGP	CH_4
GTL	679^{ab} ± 80.0	6.63^{c} ± 0.09	39.1^{ab} ± 6.61	51.5^{c} ± 10.7	156^{bc} ± 21.8	18.1^{bcd} ± 2.36
BTL	623^{abc} ± 40.8	6.74^{abc} ± 0.09	36.9^{ab} ± 4.78	89.3^{b} ± 9.18	124^{d} ± 13.8	16.5^{bcd} ± 2.48
SGTL	670^{abc} ± 53.0	6.66^{bc} ± 0.03	40.2^{a} ± 6.47	104^{b} ± 11.0	158^{b} ± 12.5	22.4^{b} ± 2.34
SBTL	641^{abc} ± 26.3	6.77^{abc} ± 0.03	41.9^{a} ± 2.01	127^{a} ± 8.62	126^{cd} ± 13.0	19.1^{bc} ± 1.85
RH	709^{a} ± 39.2	6.69^{bc} ± 0.10	40.5^{a} ± 2.53	129^{a} ± 4.00	163^{b} ± 1.12	22.1^{b} ± 0.62
RS	705^{a} ± 42.6	6.64^{c} ± 0.03	43.3^{a} ± 3.78	130^{a} ± 5.77	212^{a} ± 21.7	27.7^{a} ± 3.37
PS	534^{c} ± 69.1	6.86^{a} ± 0.03	28.5^{b} ± 3.30	142^{a} ± 4.40	86.0^{e} ± 4.82	13.7^{d} ± 1.13
BS	575^{bc} ± 60.9	6.80^{ab} ± 0.07	35.3^{ab} ± 3.15	142^{a} ± 3.47	88.8^{e} ± 5.86	13.9^{d} ± 0.35
WS	573^{bc} ± 40.2	6.77^{abc} ± 0.02	28.4^{b} ± 6.20	138^{a} ± 1.91	98.3^{de} ± 8.11	15.2^{cd} ± 1.11
SEM	32.0	0.03	2.27	3.74	6.42	0.97
P Value	P<0.01	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

Means with different letters in the same column are significantly different; SD, standard deviation; SEM, standard error of mean; GTL, green tea leaves; BTL, black tea leaves; SGTL and SBTL, green and black spent tea leaves; RH, ryegrass hay; RS, ryegrass silage; PS, paddy straws; BS, barley straws; WS, wheat straws.

of the straws. However, the ability to reduce CH_4 concentration by STL was poorer. Perhaps, this was due to lower tannins and saponins in the STL compared with the original tea leaves [1]. Tannins can reduce rumen CH_4 production [3, 5] and so saponins [8–10] by reducing protozoa and the methanogenic activitiesintherumen [8, 9].

4. Conclusion

Tea leaves have the potential as natural feed additives for ruminants to reduce rumen NH_3 but increasing potential by-pass proteins and improving the degradability of low quality straws without affecting CH_4 output. Spent tea leaves can also improve the degradability of the straws but their ability to reduce NH_3 and CH_4 concentration depends upon their tannin and saponin contents.

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