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In vitro indications for favourable non-additive effects on ruminal methane mitigation between high-phenolic and high-quality forages

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Abstract

Feeding plants containing elevated levels of polyphenols may reduce ruminal CH_4 emissions, but at the expense of nutrient utilisation. There might, however, be non-additive effects when combining high-phenolic plants with well-digestible, high-nutrient feeds. To test whether non-additive effects exist, the leaves of *Carica papaya* (high in dietary quality, low in polyphenols), *Clidemia hirta* (high in hydrolysable tannins), *Swietenia mahagoni* (high in condensed tannins) and *Eugenia aquea* (high in non-tannin phenolics) were tested alone and in all possible mixtures (*n* 15 treatments). An amount of 200 mg DM of samples was incubated *in vitro* (24 h; 39°C) with buffered rumen fluid using the Hohenheim gas test apparatus. After the incubation, total gas production, CH_4 concentration and fermentation profiles were determined. The levels of absolute CH_4, and CH_4:SCFA and CH_4:total gas ratios were lower ($P<0{\cdot}05$) when incubating a combination of *C. papaya* and any high-phenolic plants (*C. hirta*, *S. mahagoni* and *E. aquea*) than when incubating *C. papaya* alone. Additionally, mixtures resulted in non-additive effects for all CH_4-related parameters of the order of 2–15 % deviation from the expected value ($P<0{\cdot}01$). This means that, by combining these plants, CH_4 in relation to the fermentative capacity was lower than that predicted when assuming the linearity of the effects. Similar non-additive effects of combining *C. papaya* with the other plants were found for NH_3 concentrations but not for SCFA concentrations. In conclusion, using mixtures of high-quality plants and high-phenolic plants could be one approach to CH_4 mitigation; however, this awaits *in vivo* confirmation.

Key words: Ruminants: Methanogenesis: Phenolic compounds: Forage

Various investigations are currently under way to identify and test means for mitigating CH_4 emissions that originate from ruminants due to the activity of methanogenic archaea during feed fermentation[1]. As a product of fermentative digestion, CH_4 emission levels depend considerably on the quantity and composition of feeds consumed[2]. The potential of mitigating CH_4 emissions by the extracts of phenolic compounds, which are synthesised in the intermediary metabolism of plants, has been demonstrated experimentally[3–5]. Also, the direct inclusion of plants containing phenolics in ruminant diets reduced CH_4 emissions compared with control diets, both *in vitro*[6] and *in vivo*[7,8].

A major drawback in implementing diets with doses of phenolics aimed at reducing CH_4 emissions is often a decline in the digestibility of the feed and therewith the productivity of the animals[9], even at dosages where toxic side effects are excluded. As a consequence, there are often no or only small declines in CH_4 per unit of digested feed and, therefore, food produced. Analysing a larger dataset by principal components analysis illustrated that plants with high forage quality are arranged opposite to those with a high CH_4-mitigating potential[10]. This indicates that achieving both goals simultaneously is difficult. Studies by Tiemann *et al.*[8,11], where low-quality tropical *Brachiaria* hay was combined with highly tanniferous shrub forage, have shown that any reduction in CH_4 was associated with a correspondingly lower utilisation of dietary energy. This may be different when high-phenolic plants are combined with high-quality feeds. So far, studies specifically designed to measure the additivity or non-additivity of the effects (i.e. non-linear effects, elsewhere also defined as associative effects[12] when investigating combinations of plants) in the context of ruminal CH_4 emissions are scarce.

In the present study, we hypothesised that combining plants characterised by different phenolic profiles with one having high quality would have general favourable

Abbreviations: CT, condensed tannins; HT, hydrolysable tannins; NTP, non-tannin phenolics.

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non-additive effects in terms of lower CH_4 emission relative to the productivity of ruminal fermentation *in vitro*. We specifically looked at *in vitro* CH_4 emissions per unit of SCFA or total gas as indicators of fermentation productivity. For this purpose, the leaves of four tropical plants were selected as model forages. *Carica papaya* was chosen as forage of high quality. *Clidemia hirta*, *Swietenia mahagoni* and *Eugenia aquea* represented forages containing appreciable amounts of total phenolics but a different phenolic profile. These plants were selected on the basis of a previous screening experiment[10]. With this kind of experimental design, it is possible to demonstrate *in vitro* the non-additivity of the properties of differing forages, which in the best case can be used as a first step towards developing forage-based diets with lowered methanogenic potential without similarly impaired ruminal fermentation efficiency. However, with this approach, it is not possible to distinguish whether the resulting effects are dose–response effects of any single compound or interaction effects of different compounds. Although *in vitro* evidence has a limited applicability for the situation *in vivo*, it provides additional information about the occurrence of such complementary effects of feeds with differing profiles in plant secondary compounds on nutrition processes in ruminants, which have already been described *in vivo* in another context[13]. The present study aimed to indicate areas on which future *in vivo* experiments could focus.

Materials and methods

Experimental design and plant material

All plant samples were incubated both individually and in all possible mixtures (Table 1). The mixtures consisted of two, three or four plants represented in equal proportions. This resulted in a total of fifteen treatments. The leaves of *C. papaya* were characterised by high crude protein contents, low contents of fibre and lignin as well as favourably high *in vitro* organic matter digestibility[10]. This differed clearly from the properties of the three high-phenolic plants selected that were rich in phenolics (7- to 9-fold levels of total phenolics compared with *C. papaya*). Concerning the phenolic profile, *C. hirta* is rich in hydrolysable tannins (HT), *S. mahagoni* contains particularly high levels of condensed tannins (CT) as well as appreciable levels of HT and non-tannin phenolics (NTP), and *E. aquea* is especially rich in NTP and lignin. The selected plants were considered to be suitable models for the purpose of comparing plants with quite similar contents but different categories of phenolic compounds. Even though not very common globally and not used as the sole feed, the leaves of *C. papaya*, *C. hirta*, *S. mahagoni* and *E. aquea* are used either as ruminant feeds in rural areas (*C. papaya* and *C. hirta*) or as traditional veterinary medicinal plants in the areas around Bogor on the Java island of Indonesia[10]. Since the present study aimed at the basic question of non-additivity when combining single forages instead of formulating complex diets, no standardisation was applied for other nutrients such as crude protein or neutral-detergent fibre, although this might have interfered with the effects of phenolic compounds.

Table 1. Treatment formulation and amounts (mg DM) of plants incubated *in vitro*

Treatments	P	C	S	E
P	200	–	–	–
C	–	200	–	–
S	–	–	200	–
E	–	–	–	200
PC	100	100	–	–
PS	100	–	100	–
PE	100	–	–	100
CS	–	100	100	–
CE	–	100	–	100
SE	–	–	100	100
PCS	67	67	67	–
PCE	67	67	–	67
PSE	67	–	67	67
CSE	–	67	67	67
PCSE	50	50	50	50

P, *Carica papaya*; C, *Clidemia hirta*; S, *Swietenia mahagoni*; E, *Eugenia aquea*.

The leaves of the experimental plants were collected in November 2008 from the area of the Indonesian Research Institute for Animal Production, Ciawi, Bogor, located at an elevation of 350 m above sea level. About 3 kg fresh matter of each plant species was sampled. Each sample consisted of leaves from several individual plants from the same species. The samples were immediately air-dried in a greenhouse for 2 d, followed by oven-drying overnight at 50°C. After drying, the samples were ground to pass a 1 mm sieve, and then subjected to chemical analysis and *in vitro* incubation. As the same batches of the four feeds as those tested in an earlier study were used in the present study, no new compositional analyses were performed and the analytical procedures have been described in detail in Jayanegara *et al.*[10]. Briefly, for *C. papaya*, *C. hirta*, *S. mahagoni* and *E. aquea*, the following concentrations (g/kg DM) were analysed: crude protein, 386, 129, 112 and 199; neutral-detergent fibre, 155, 232, 281 and 479; total extractable phenolics, 25, 216, 207 and 169; total tannins, 8, 212, 138 and 67; CT, 0, 10, 86 and 40; HT, 8, 202, 52 and 27; NTP, 17, 4, 69 and 102[10].

In vitro *procedure and analyses*

An amount of 200 mg DM of individual plants or mixtures was incubated with 10 ml of rumen fluid and 20 ml of buffer solution using the Hohenheim gas test apparatus[14] with modified syringes[15]. The latter have two outlets, in which the first outlet is designed for filling and emptying the liquid phase and the second allows sampling from the gas phase. Incubation was carried out for 24 h at 39°C in four subsequent runs, comprising two treatment replicates per run (n 8). This was complemented for each run by three syringes without feed, with standard hay and concentrate. Both standard hay and concentrate were obtained from the Institute of Animal Nutrition, University of Hohenheim, Stuttgart, Germany. Data on the expected amounts of total gas produced from incubation of the standards were used in comparison with those actually measured, in order to monitor whether incubation

went in a normal way and to adjust total gas[14]. The donor of rumen fluid was a rumen-cannulated lactating Brown Swiss cow; the fluid was taken before the morning feeding. In order to prevent any previous adaptive processes to the phenolics, the cow received hay made from a ryegrass–white-clover ley with *ad libitum* access and 0·5 kg/d of dairy cow concentrate (UFA 149, UFA AG). The cow was cared for according to the Swiss guidelines for animal welfare. After collection, rumen fluid was strained through four layers of gauze (1 mm pore size, Type 17; MedPro Novamed AG) in order to filter out any feed particles.

The volume of fermentation gas produced during 24 h of incubation was read from the calibrated scale on each glass syringe. The liquid phase in each syringe was decanted. Subsequently, 0·15 ml of fermentation gas were withdrawn with a Hamilton syringe (Hamilton AG) through a gas-tight septum covering the outlet. This gas sample was injected into a gas chromatograph (Model 5890 Series II; Hewlett Packard) for measuring CH_4 and H_2 concentrations. NH_3 and pH of the incubation liquid were determined with a potentiometer (Model 632; Metrohm) equipped with the corresponding electrodes. Total NH_3 was calculated from NH_3 concentration and the volume of the incubation liquid. SCFA were analysed using HPLC (LaChrom, L-7000 series; Hitachi Limited) equipped with an UV–VIS detector, read at 210 nm[10]. Total viable bacterial and protozoal numbers were counted by direct microscopic counting using Bürker counting chambers (Blau Brand) with depths of 0·02 and 0·1 mm, respectively. For bacterial counting, samples were treated with Hayem solution ($HgCl_2$, 2·5 mg/ml; Na_2SO_4, 25 mg/ml; NaCl, 5·0 mg/ml). Viability of bacteria was accounted for by counting only moving individuals. Before protozoal counting, samples were treated with 1:10 diluted formalin (40/100, w/v in water). Only intact protozoa and no fragments were counted.

Calculations and statistical analysis

Following Menke & Steingass[14], gas production from the blank was subtracted from all samples incubated to obtain the net gas production. Subsequently, gas production from the hay standard (44·43 ml gas/200 mg DM; 24 h incubation) was divided by the measured net value of the hay standard to provide the correction factor F_H. Similarly, gas production from the concentrate standard (65·18 ml/200 mg DM; 24 h incubation) was divided by the measured net gas production of the concentrate standard to provide F_C. The average value of F_H and F_C was used for the adjustment. Data on CH_4 and H_2 concentrations were transformed into volume (ml, μl) data by multiplying the measured concentrations with the measured total gas volume. To get a direct meaningful relationship of CH_4 to SCFA, molar amounts were calculated by assuming a density of 0·67 kg/m^3 for CH_4 gas at 1·013 bar and 16°C[16].

The data generated were subjected to a mixed model of ANOVA. Incubation runs, serving as a block in the ANOVA model, were considered as random effects, while plant treatments were included as fixed effects. If fixed effects were significant at $P<0·05$, multiple comparisons among means were made using Tukey's *post hoc* test. Before ANOVA, bacterial and protozoal counts were transformed into their logarithmic units. All statistical analyses were performed using SPSS statistical software version 17.0[17].

The main purpose of the experiment was to reveal the presence of non-additive effects. Non-additive effects were defined as the deviations of the observed values (obtained by measurements) from the expected values (calculated as arithmetic means of the values obtained by the respective individual plant incubations). These differences were analysed for all plant mixtures using a paired *t* test and presented as (observed value − expected value)/expected value × 100 % following Niderkorn *et al.*[12]. All expected ratio values were calculated by the actually measured values for each individual incubation. This means that the expected values were not calculated from the already averaged ratio values, as shown in Tables 2 and 3. For the ratio of CH_4:total gas (ml/l), the expected value was calculated as $((CH_4\ plant_1 + CH_4\ plant_{i-n})/n)/((\text{total gas plant}_1 + \text{total gas plant}_{i-n})/n)$, where n is the number of involved plant species. The same principle was applied for calculating the expected value for the ratio of CH_4:SCFA (mmol/mol).

Results

Effects of plants and combinations of plants on in vitro fermentation

The total SCFA amount was superior when *C. papaya* was incubated compared with the other three plants ($P<0·05$), where *E. aquea* was inferior to *C. birta* and *S. mahagoni* (Table 2). The acetate:propionate ratio was shifted towards acetate when *E. aquea* was incubated compared with the other three forages ($P<0·05$). Combining the different forages resulted in values for SCFA production and proportions of acetate and propionate being always intermediate between those obtained from individual incubations. For the molar proportions of butyrate, valerate and their *iso* forms, the effects were less clear.

Total gas produced from incubation with *C. papaya* was twice that with *C. birta* and *S. mahagoni* ($P<0·05$), and the latter two plants produced twice as much total gas compared with *E. aquea* ($P<0·05$; Table 3). The volumes of CH_4 and H_2 (which on average amounted to only about 0·1 % of that of CH_4) varied, when total gas varied. Nevertheless, the gas composition differed among the treatments. Incubating *S. mahagoni* led to the lowest CH_4:total gas ratio among the individual plants ($P<0·05$), while incubating *C. papaya* produced twice the level. The lowest H_2:total gas proportion was found when *E. aquea* (40 μl/l) was incubated and the highest with *C. papaya* (110 μl/l; $P<0·05$; data not shown). The amounts of CH_4 in relation to the amounts of SCFA after incubation differed ($P<0·05$) between each of the forage species, and were ranked in the order of *C. papaya* > *C. birta* > *S. mahagoni* > *E. aquea*. The concentration of NH_3 in the incubation liquid was highest when *C. papaya* was incubated alone, whereas the ratio of NH_3-N:dietary N was highest with *E. aquea* incubation and differed ($P<0·05$)

Table 2. Effect of plant species or species combinations on *in vitro* incubation liquid SCFA profiles (observed values, *n* 8)

		Molar proportion of total SCFA						
Treatments*	Total SCFA (mmol/l)	C_2	C_3	C_4	*iso*C_4	C_5	*iso*C_5	C_2:C_3
P	$75{\cdot}7^{i}$	$70{\cdot}9^{a}$	$16{\cdot}7^{d}$	$8{\cdot}6^{b,c}$	$1{\cdot}43^{c}$	$1{\cdot}24^{c}$	1·09	$4{\cdot}36^{a}$
C	$50{\cdot}0^{b,c}$	$73{\cdot}6^{b,c,d}$	$15{\cdot}4^{a,b,c}$	$9{\cdot}0^{c}$	$0{\cdot}59^{a,b}$	$0{\cdot}52^{a,b}$	0·80	$4{\cdot}79^{a,b,c}$
S	$50{\cdot}4^{b,c,d}$	$74{\cdot}5^{c,d}$	$16{\cdot}3^{b,c,d}$	$6{\cdot}8^{a}$	$0{\cdot}42^{ab}$	$0{\cdot}91^{b,c}$	1·02	$4{\cdot}60^{a,b}$
E	$42{\cdot}7^{a}$	$74{\cdot}9^{d}$	$14{\cdot}3^{a}$	$8{\cdot}5^{b,c}$	$0{\cdot}53^{a,b}$	$0{\cdot}63^{a,b}$	1·16	$5{\cdot}26^{c,d}$
PC	$60{\cdot}5^{g,h}$	$72{\cdot}0^{a,b}$	$16{\cdot}6^{c,d}$	$8{\cdot}6^{b,c}$	$0{\cdot}82^{a,b}$	$0{\cdot}67^{a,b}$	1·21	$4{\cdot}34^{a}$
PS	$62{\cdot}3^{h}$	$73{\cdot}0^{a,b,c,d}$	$16{\cdot}4^{c,d}$	$8{\cdot}4^{b,c}$	$0{\cdot}85^{a,b,c}$	$0{\cdot}66^{a,b}$	0·80	$4{\cdot}47^{a,b}$
PE	$57{\cdot}4^{f,g}$	$72{\cdot}7^{a,b,c,d}$	$16{\cdot}2^{b,c,d}$	$8{\cdot}6^{b,c}$	$0{\cdot}80^{a,b}$	$0{\cdot}74^{a,b}$	0·97	$4{\cdot}51^{a,b}$
CS	$50{\cdot}9^{b,c,d}$	$74{\cdot}5^{c,d}$	$16{\cdot}0^{b,c,d}$	$7{\cdot}5^{a,b}$	$0{\cdot}53^{a,b}$	$0{\cdot}56^{a,b}$	0·80	$4{\cdot}67^{a,b,c}$
CE	$47{\cdot}0^{a,b}$	$73{\cdot}9^{b,c,d}$	$14{\cdot}5^{a}$	$9{\cdot}3^{c}$	$0{\cdot}66^{a,b}$	$0{\cdot}42^{a}$	1·19	$5{\cdot}11^{c,d}$
SE	$46{\cdot}9^{a,b}$	$74{\cdot}5^{c,d}$	$15{\cdot}4^{a,b,c}$	$8{\cdot}0^{a,b,c}$	$0{\cdot}62^{a,b}$	$0{\cdot}57^{a,b}$	0·92	$4{\cdot}84^{b,c,d}$
PCS	$57{\cdot}5^{f,g}$	$72{\cdot}7^{a,b,c}$	$16{\cdot}1^{b,c,d}$	$8{\cdot}8^{b,c}$	$1{\cdot}03^{b,c}$	$0{\cdot}55^{a,b}$	0·88	$4{\cdot}53^{a,b}$
PCE	$54{\cdot}8^{d,e,f}$	$72{\cdot}7^{a,b,c}$	$16{\cdot}0^{b,c,d}$	$9{\cdot}0^{c}$	$0{\cdot}62^{a,b}$	$0{\cdot}61^{a,b}$	1·16	$4{\cdot}56^{a,b}$
PSE	$56{\cdot}4^{e,f,g}$	$72{\cdot}6^{a,b,c}$	$15{\cdot}9^{b,c,d}$	$9{\cdot}1^{c}$	$0{\cdot}84^{a,b,c}$	$0{\cdot}48^{a}$	1·17	$4{\cdot}58^{a,b}$
CSE	$47{\cdot}7^{b}$	$74{\cdot}2^{b,c,d}$	$15{\cdot}1^{a,b}$	$8{\cdot}5^{b,c}$	$0{\cdot}71^{a,b}$	$0{\cdot}61^{a,b}$	0·90	$4{\cdot}93^{b,c,d}$
PCSE	$52{\cdot}9^{c,d,e}$	$73{\cdot}9^{b,c,d}$	$15{\cdot}7^{a,b,c,d}$	$8{\cdot}6^{b,c}$	$0{\cdot}39^{a}$	$0{\cdot}48^{a}$	0·90	$4{\cdot}71^{a,b,c}$
SEM	0·83	0·16	0·11	0·10	0·039	0·028	0·044	0·038
P	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	0·449	<0·001

C_2, acetate; C_3, propionate; C_4, butyrate; C_5, valerate; C_2:C_3, acetate:propionate ratio.
[a-i] Mean values within a column with unlike superscript letters were significantly different ($P<0{\cdot}05$).
* C, *Clidemia hirta*; E, *Eugenia aquea*; P, *Carica papaya*; S, *Swietenia mahagoni*.

from the values measured with the other plants. There were some differences in the pH of the incubation liquid, but not ($P>0{\cdot}05$) in the logarithmic counts of viable bacteria and protozoa across all experimental treatments (data not shown).

In general, combining either *C. hirta*, *S. mahagoni* or *E. aquea* with *C. papaya* in binary mixtures resulted in lower total gas production compared with the incubation of *C. papaya* alone ($P<0{\cdot}05$). CH_4-related variables (CH_4, CH_4:SCFA and CH_4:total gas) were also reduced by mixing these plants ($P<0{\cdot}05$) compared with the incubation of *C. papaya* alone. The mixing of *C. hirta* and/or *E. aquea* with *S. mahagoni* resulted in a lower ($P<0{\cdot}05$) CH_4:total gas ratio than all mixtures including *C. papaya*. Furthermore, all plant mixtures caused a decrease in the ratio of CH_4:SCFA compared with pure *C. papaya* incubation ($P<0{\cdot}05$). The lowest ratios were found with the mixtures containing no *C. papaya*; however, this resulted in a very low level of SCFA production. The mixtures containing *C. papaya* increased ruminal NH_3 concentrations compared with the mixtures without this plant.

Non-additive effects of using plant mixtures

No non-additive effects on total SCFA concentration were observed when combining any plant species (Table 4). Binary mixtures including *C. papaya* did not result ($P>0{\cdot}05$) in non-additive effects on total gas production. However, when *C. papaya* was combined with two or all three high-phenolic plants, non-additive effects ($P<0{\cdot}05$) on gas production were observed. Combining *C. papaya* with any

Table 3. Effect of plant species or species combinations on *in vitro* rumen fermentation measurements (observed values, *n* 8)

Treatments*	Total gas (ml)	CH_4 (ml)	H_2 (μl)	CH_4:total gas (ml/l)	CH_4:SCFA (mmol/mol)	pH	NH_3 (mmol/l)	NH_3-N:dietary N (mg/mg)
P	$44{\cdot}3^{g}$	$8{\cdot}1^{i}$	$5{\cdot}1^{f}$	181^{i}	149^{j}	$7{\cdot}38^{c,d}$	$27{\cdot}3^{h}$	$0{\cdot}93^{c,d}$
C	$22{\cdot}1^{c}$	$2{\cdot}5^{e}$	$1{\cdot}8^{c,d}$	$112^{b,c}$	$69^{d,e}$	$7{\cdot}20^{a}$	$8{\cdot}8^{a,b}$	$0{\cdot}90^{c}$
S	$22{\cdot}7^{c,d}$	$2{\cdot}1^{c,d,e}$	$1{\cdot}2^{a,b,c}$	91^{a}	$57^{b,c}$	$7{\cdot}31^{b,c}$	$8{\cdot}4^{a}$	$0{\cdot}98^{c,d}$
E	$10{\cdot}4^{a}$	$1{\cdot}3^{a}$	$0{\cdot}4^{a}$	$122^{d,e}$	42^{a}	$7{\cdot}34^{c}$	$10{\cdot}7^{c,d}$	$1{\cdot}41^{e}$
PC	$33{\cdot}1^{f}$	$4{\cdot}7^{h}$	$3{\cdot}0^{e,f}$	143^{g}	109^{i}	$7{\cdot}34^{c}$	$14{\cdot}1^{f,g}$	$0{\cdot}72^{a}$
PS	$32{\cdot}6^{f}$	$4{\cdot}5^{h}$	$2{\cdot}9^{e,f}$	$137^{f,g}$	100^{h}	$7{\cdot}32^{b,c}$	$13{\cdot}5^{e,f}$	$0{\cdot}71^{a}$
PE	$27{\cdot}9^{e}$	$4{\cdot}5^{h}$	$2{\cdot}2^{d,e}$	159^{h}	$107^{h,i}$	$7{\cdot}38^{c,d}$	$15{\cdot}8^{g}$	$0{\cdot}86^{b,c}$
CS	$21{\cdot}5^{c}$	$2{\cdot}3^{d,e}$	$1{\cdot}1^{a,b,c}$	104^{b}	$61^{c,d}$	$7{\cdot}26^{a,b}$	$8{\cdot}3^{a}$	$0{\cdot}90^{c}$
CE	$15{\cdot}3^{b}$	$1{\cdot}8^{b,c}$	$1{\cdot}5^{b,c,d}$	$115^{c,d}$	$53^{b,c}$	$7{\cdot}32^{b,c}$	$9{\cdot}1^{a,b,c}$	$1{\cdot}05^{d}$
SE	$15{\cdot}9^{b}$	$1{\cdot}7^{a,b}$	$0{\cdot}9^{a,b,c}$	103^{b}	$49^{a,b}$	$7{\cdot}34^{c}$	$8{\cdot}5^{a,b}$	$1{\cdot}05^{d}$
PCS	$28{\cdot}3^{e}$	$3{\cdot}6^{g}$	$1{\cdot}6^{b,c,d}$	$127^{e,f}$	87^{g}	$7{\cdot}33^{b,c}$	$10{\cdot}3^{b,c,d}$	$0{\cdot}65^{a}$
PCE	$24{\cdot}1^{d}$	$3{\cdot}5^{g}$	$1{\cdot}0^{a,b,c}$	142^{g}	87^{g}	$7{\cdot}38^{c,d}$	$12{\cdot}0^{d,e}$	$0{\cdot}77^{a,b}$
PSE	$24{\cdot}5^{d}$	$3{\cdot}3^{f,g}$	$1{\cdot}3^{a,b,c,d}$	$132^{e,f}$	$80^{f,g}$	$7{\cdot}35^{c,d}$	$11{\cdot}4^{d}$	$0{\cdot}76^{a,b}$
CSE	$16{\cdot}9^{b}$	$1{\cdot}9^{b,c,d}$	$0{\cdot}9^{a,b,c}$	$111^{b,c}$	$55^{b,c}$	$7{\cdot}33^{b,c}$	$9{\cdot}0^{a,b,c}$	$1{\cdot}04^{d}$
PCSE	$22{\cdot}9^{c,d}$	$2{\cdot}9^{f}$	$0{\cdot}7^{a,b}$	$128^{e,f}$	$77^{e,f}$	$7{\cdot}42^{d}$	$10{\cdot}3^{b,c,d}$	$0{\cdot}75^{a,b}$
SEM	0·78	0·16	0·44	2·4	5·1	0·01	0·47	0·022
P	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001

[a-j] Mean values within a column with unlike superscript letters were significantly different ($P<0{\cdot}05$).
* C, *Clidemia hirta*; E, *Eugenia aquea*; P, *Carica papaya*; S, *Swietenia mahagoni*.

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Table 4. Non-additivity of the effects of the plant mixtures (difference in observed values to expected values, in percentage of the expected values†) on *in vitro* rumen fermentation parameters (*n* 8)

Treatments‡	Total SCFA	Total gas	CH_4	CH_4:SCFA	CH_4:total gas	pH	NH_3	NH_3-N:dietary N
PC	−3·1	−0·5	−10·1**	−6·9	−9·6***	0·6*	−22·3**	−22·2***
PS	−0·4	−3·0	−12·1***	−10·9*	−9·4***	−0·3	−24·3**	−25·8***
PE	−2·5	1·9	−5·2**	−2·2	−7·0**	0·3	−16·8**	−27·0***
CS	1·6	−4·1*	−1·4	−2·7	2·9	0·0	−4·0	−4·7
CE	1·8	−5·8	−6·0	−7·7*	0·0	0·7	−6·8	−9·8
SE	1·1	−4·3**	−1·4	−2·3	3·0	0·2	−11·4*	−12·3*
PCS	−1·5	−4·5**	−14·5***	−12·8**	−10·4***	0·4	−31·0***	−31·5***
PCE	−1·8	−5·6*	−12·5**	−10·5*	−7·3***	0·9*	−23·7***	−29·2***
PSE	0·6	−5·3**	−15·0***	−15·2**	−10·3***	0·0	−26·3***	−32·0***
CSE	0·1	−8·5**	−3·7	−3·7	5·4	0·6**	−3·2	−5·3
PCSE	−2·8	−7·5*	−15·4***	−12·6**	−8·3***	1·5**	−25·8***	−29·8***

* $P<0·05$, ** $P<0·01$, *** $P<0·001$.
† Mean values of the individual plants present in the mixtures incubated individually.
‡ C, *Clidemia hirta*; E, *Eugenia aquea*; P, *Carica papaya*; S, *Swietenia mahagoni*.

of the other plants (*C. hirta*, *S. mahagoni* and *E. aquea*) led to non-additive effects (at least at $P<0·01$) in terms of CH_4 emission, either when expressed as the absolute CH_4 amount or as the CH_4:SCFA and CH_4:total gas ratios. Non-additive effects, apparent as negative deviations from the values predicted when assuming additive responses to the plant combinations, were observed for mixtures of two, three or four plants, and ranged from 5 to 15%, 2 to 15% and 7 to 10% for CH_4, CH_4:SCFA and CH_4:total gas, respectively. In contrast, no non-additive effects ($P>0·05$) on CH_4 emissions were found when combining the high-phenolic plants only, i.e. *C. hirta*, *S. mahagoni* and *E. aquea*, in 2- or 3-fold mixtures. For the molar amount of CH_4 produced per molar amount of SCFA synthesised, all multiple combinations comprising *C. papaya* resulted in non-additive effects (at least at $P<0·05$). For the binary combinations, such non-additive effects were found only for *C. papaya* together with *S. mahagoni* and for *C. hirta* together with *E. aquea* ($P<0·05$). With regard to NH_3-related variables, non-additive effects (negative deviation; $P<0·01$) of all mixtures of *C. papaya* combined with the other plants were observed for NH_3 and NH_3-N:dietary N. The magnitude of the effects was considerable with deviations of mostly more than −20%. No such effect was found with combinations of the high-phenolic plants, except for the mixture of *S. mahagoni* and *E. aquea*.

Discussion

In natural environments, ruminants select diets from various forage resources such as grasses, forbs, shrubs and tree leaves. These plants may largely vary in their nutritional composition such as energy, protein, vitamins and minerals, and in contents of plant secondary metabolites. Under such conditions, interactions between different kinds of forages and chemical constituents may occur, which might influence intake behaviour, digestion, well-being and performance[13,18]. Niderkorn & Baumont[19] described that mixing two or more different forages can even result in a different response for various parameters (higher or smaller) from that expected if just considering the average of the effects of the individual plants. Yet few studies have specifically investigated the non-additive effects of dietary ingredients, or plants characterised by specific compounds, on rumen fermentation and digestibility *in vitro*[20,21] and *in vivo*[22,23]. The results reported so far are quite variable; some mixtures showed non-additive effects (either favourable or unfavourable) and others were simply additive.

There is still particularly little information available on the non-additive effects of mixed feeds on CH_4 emissions[24,25]. Even less literature is available for mixtures involving plants differing in phenolic profiles[12]. This is of a particularly high interest as feeds rich in total phenolics cannot be fed alone and it depends on the nature of the relationship between compounds whether their basic anti-methanogenic potential[26,27] is enforced, unchanged or decreased by the combination with plants of high forage quality. In this context, a favourable non-additive effect would mean that the combination of a high-phenol 'plant X' with a high-quality 'plant Y' would reduce the anti-methanogenic potential coming from plant X to a proportionately lesser extent than the feeding value of plant Y. This concept is simplistic in a way because it cannot answer the question whether any non-additive effects found are based on a non-linear dose–response relationship with a single compound or whether they emerge from an interaction of various plant compounds. However, even though non-linearity cannot be traced back to the level of compounds involved (which may also include secondary compounds other than phenolics), this perspective might indicate the potential of complementarity on the level of forages, which is the relevant level in practical livestock feeding, particularly in smallholder farms of developing tropical countries.

Non-additive effects of plant mixtures on ruminal methane formation in relation to the level of ruminal fermentation

Phenolics have been shown to reduce the population of methanogenic archaea in the rumen[4] and, therefore, to mitigate CH_4 emissions. In addition, phenolics interact with other chemical plant constituents such as protein and carbohydrates (both fibre and non-fibre carbohydrates) via hydrogen

bonds[28]. Inhibition of carbohydrate digestion leads to a lower formation of H_2, which is a substrate for methanogenesis[29]. If this is the major way to mitigate CH_4 emissions, there is no advantage in implementing this supplementation strategy into practical feeding as productivity of the animals is concomitantly hampered. The addition of high-phenolic plants to high-quality forages might result in an even larger depression in feed utilisation as nutrients of an inherently higher digestibility might be transformed into indigestible compounds. This was confirmed in the present study by the adverse non-additive effects found with some mixtures in total gas production. However, such unfavourable non-additive effects mainly occurred for mixtures containing *S. mahagoni*, which is rich in CT content (numerically also for the mixture with *C. papaya*). Apart from the inhibitory effects of CT on the growth and activity of rumen micro-organisms and the enzymes secreted[30], a stable complex between CT and other chemical constituents[11,31] might explain non-additive effects which decrease fermentative activity in combinations including *S. mahagoni*. The SCFA amounts did not show this effect even in combinations including *S. mahagoni*. This indicates that the hypothesis that combinations with high-phenolic forages cause non-additive decreases in ruminal productivity is neither fully supported nor disproved by the present results. However, the NTP in *E. aquea* are likely to possess no or a smaller binding capability. Also, complexes with HT are degradable under ruminal conditions[30], which is relevant for *C. hirta* being rich in HT. However, it should be noted that *C. hirta*, when fed at a high proportion (0·5 parts of the total ration; air-dry basis), may lead to hepato- and nephrotoxicity and be associated with gastroenteritis in goats as HT may be absorbed[32].

Concerning CH_4 production relative to total gas or SCFA, substantial non-additive effects were found when incubating *C. papaya* together with the high-phenolic plants or their mixtures (not significant for some binary combinations concerning SCFA). Even though there could be a certain bias in total gas, as some of the gas could be CO_2 released from the buffer, both variables pointed to the same direction and thus indicate that methanogenesis was generally more decreased than fermentation productivity when adding the high-phenolic plants to *C. papaya*. It appears that, in combination with a high-quality plant, the phenolic compounds inhibit CH_4 production more than expected but not at the cost of a more than proportionate impairment of ruminal productivity. In comparison with a high-phenolic plant, a high-quality plant containing low levels of phenolics might not be affected as much in the forage value, whereas the phenolics are still able to exert their direct anti-methanogenic property. This means that the stoichiometry of CH_4 formation might be affected. If CH_4 formation decreases more than the amounts of either total gas or SCFA, both indicating fermentative productivity, there has to be an alternative sink for the emerging H_2. The H_2 concentrations in the present study were lower by a factor of 1000 compared with CH_4, which is in accordance with other studies and makes it generally difficult to explain alterations in the concentration of CH_4 with those in H_2[33]. The concentration of H_2 was never increased in treatments with negative non-additive effects on CH_4 traits. For the combination with the strongest non-additive effects on CH_4 (*C. papaya*, *S. mahagoni* and *E. aquea*), the amount of gaseous H_2 also underwent a negative non-additive effect. Thus, there seemed to have been generally less H_2 present in the incubation unit. Lower ruminal H_2 concentrations can be a consequence of increased ruminal propionate formation[33]. However, this was not the case with the treatments discussed here. A shortcoming of the present study is that the concentrations of nutrients serving as fermentation substrates alter too much between the different forage combinations and thus do not allow for clear stoichiometric comparisons. Furthermore, non-phenolic plant secondary compounds, such as papain in the papaya leaves, that have not been measured could have interfered. Although the reasons for this disparity of non-additive effects remain unclear, the results of the present study might offer opportunities to develop diets that are concomitantly effective for production and CH_4 mitigation.

According to Niderkorn *et al.*[12] and Robinson *et al.*[21] non-additive effects of feeds on total gas production *in vitro* may even be clearly more pronounced after a shorter incubation time of 3·5 or 8 h, when compared with 24 h. A lack of significant non-additive effects on CH_4 at 24 h of incubation was also observed by Goel *et al.*[34]. In that study, combining different levels of the leaves from *Carduus pycnocephalus*, a plant containing phenolics in unknown concentration, with hay or concentrate did not lead to differences between the observed and expected values for *in vitro* CH_4 emissions. The time effect, however, was not measured in the present study, and all effects found were present after 24 h.

Almost no non-additive effects were found for mixtures of the high-phenolic plants except with regard to CH_4:total gas and CH_4:SCFA. Hypothetically, non-additive effects for mixtures of plants containing high concentrations of different phenols could be significant in two extreme cases: either the effects of different phenolics are mutually strengthening their activity, which would result in less CH_4 than expected from incubating individual feeds, or they would counterbalance each other, which would result in the opposite. The response pattern in non-additive effects on CH_4 found in the present study suggests that such effects are stronger when combining individual plants that are distinctly different in their general CH_4 production potential than when combining forages containing similarly high levels of potentially CH_4-inhibiting constituents. The latter plants already produced low CH_4 emissions (2·5, 2·1 and 1·3 ml CH_4/200 mg DM with *C. hirta*, *S. mahagoni* and *E. aquea*, respectively, when incubated alone. This favourably compares with the levels of 2·7, 1·6 and 1·0 ml CH_4/200 mg DM described earlier in Jayanegara *et al.*[10]). In both experiments, this was far below the level found with *C. papaya* (8·1 and 7·4 ml CH_4/200 mg DM, respectively). Even though the high-phenolic plants differed in their phenolic profiles, CH_4 production potential seems to be especially determined by total phenolic contents rather than by the specific phenolic fraction (NTP, CT or HT)[10] or other potentially effective compounds.

Generally, the incubations resulted in SCFA concentrations and a pH that are comparable with other experiments incubating high-phenolic feeds[10,35]. The resulting pH was high, but

according to Van Kessel & Russell[36], this should not have impaired methanogenesis. However, since the pH in the rumen is expected to be clearly lower *in vivo*, this is one factor which makes it necessary to confirm the results also in *in vivo* experiments.

Non-additive effects of plant mixtures on ruminal ammonia formation

The non-additive effects of the plant mixtures on NH_3 variables followed the pattern found with the CH_4-related variables, i.e. they were significant and negative for the mixtures containing *C. papaya*, and even to a higher magnitude than the CH_4 variables. Again, the largely contrasting NH_3-generating properties between *C. papaya* (27 mmol/l) and the plants characterised by high total phenolics (ranging from 8 to 11 mmol/l) might have opened room for generating non-additive effects. Mixing forages with high levels of ruminally degradable protein with those elevated in phenolics could therefore be particularly useful to prevent excessive degradation of protein into NH_3 as non-additive effects enhance this process. The different groups of phenolics have different protein-binding capacities. While NTP, by definition, do not bind proteins, this is different with both HT and CT, and the bonds formed with CT are particularly resistant[30]. However, in the present study, only limited non-additive effects on NH_3 production were found when incubating the plants with different types of phenolics alone when compared with incubations of the mixtures with *C. papaya*.

The general presence of the non-additive effects of plant combinations with different phenolic profiles might also be reflected by the fact that combinations of shrub species that contained different classes of plant secondary metabolites enhanced intake by ruminants[37–39]. This could be explained by the attempt to achieve a better nutrient balance, to find the optimum medicinal benefit and to minimise the harmful effects of each of the toxins[13,37,38,40].

Conclusions

Combining plants containing phenolics with the high-quality leaves of *C. papaya* reduced ruminal CH_4 emissions more than predicted from the arithmetic means. This was independent of the respective phenolic profile. These non-additive effects of plant combinations were also apparent in the ratio of CH_4:SCFA. Provided the confirmation of such effects *in vivo*, this could mean that such mixtures of high-quality and high-phenolic forages could help to mitigate CH_4 without correspondingly extensively reducing ruminal nutrient utilisation. The mixtures would also prevent excessive degradation of protein into NH_3. Whether or not non-additive effects of combining such plants can be recovered *in vivo* and on which compound interactions they mechanistically rely merits further studies.

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