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Influence of Apple and Citrus Pectins, Processed Mango Peels, a Phenolic Mango Peel Extract, and Gallic Acid as Potential Feed Supplements on in Vitro Total Gas Production and Rumen Methanogenesis

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Supporting Information

ABSTRACT: Several food processing byproducts were assessed as potential feed and feed supplements. Since their chemical composition revealed a high nutritional potential for ruminants, the Hohenheim in vitro gas test was used to investigate total gas, methane, and volatile fatty acid production as well as protozoal numbers after ruminal digestion of different substrate levels. Processing byproducts used were low- and high-esterified citrus and apple pectins, integral mango peels, and depectinized mango peels. In addition, the effect of a phenolic mango peel extract and pure gallic acid was investigated. The highest decrease in methane production (19%) was achieved by supplementing high levels of low-esterified citrus pectin to the hay-based diet. Interestingly, total gas production was not affected at the same time. Showing valuable nutritional potential, all byproducts exhibited, e.g., high metabolizable energy (11.9−12.8 MJ/kg DM). In conclusion, all byproducts, particularly low-esterified citrus pectin, revealed promising potential as feed and feed supplements.

KEYWORDS: *methane, Hohenheim gas test, phenolic compounds, volatile fatty acids, protozoa, ruminant feed, byproducts, food waste*

INTRODUCTION

Byproducts of fruit and vegetable processing, in particular fruit peels, are a cheap and valuable source of phytochemicals having high potential as functional bioactives.[1] In the European Community, annually more than 150 million tons of waste materials accrue from plant food processing. They are disposed at the expense of the processors, although they could benefit from selling valuable byproducts as feed or feed supplements.[2] Therefore, byproducts need to fulfill the requirements of providing nutrients and an acceptable metabolizable energy. Such byproducts might be of particular interest for sustainable agricultural practices, if the production of greenhouse gases like methane by livestock could be reduced at the same time.

Agriculture accounts for 10 to 12% of total greenhouse gas emissions and the livestock sector for 37% of the global anthropogenic methane emissions, as methanogenic microorganisms in the digestive system of ruminants reduce carbon dioxide (CO_2) by hydrogen (H_2), yielding methane and water. During the past decade, methane production by agriculture and especially by ruminants has received global attention due to its significant contribution by about 50 to 80% of agricultural methane emissions to greenhouse gas emissions.[3,4] Besides the ecological impact, energy loss resulting from methane formation causes an energy deficit of about 2 to 15% of the gross energy in feed.[5] However, methane formation represents a physiologically important pathway to avoid hydrogen accumulation in the rumen, which would inhibit the dehydrogenase activity catalyzing the oxidation of reduced cofactors (NADH, NADPH, FADH), i.e., the regeneration of the reoxidized counterparts NAD^+, $NADP^+$, and FAD^+.[6]

Reduction of methane emission from ruminants has been subject of numerous studies and was summarized by Eckard et al.[7] For instance, vaccination against methanogens may be an option to achieve this goal. Additionally, methane-suppressing properties of the bacteriocins biovicin HC5 and nisin, respectively, were demonstrated. Furthermore, a variable influence of several chemical inhibitors such as brominated compounds on methane release was shown. Besides exogenous additives, the composition of the ruminant diet was shown to exert substantial influence on methanogenesis. For instance, digestible fibrous carbohydrates were shown to promote methanogenesis more intensively than soluble carbohydrates and proteins.[8,9] In contrast, addition of oils to diets has been shown to reduce methane production, methanogens, and protozoa.[10]

Ruminal protozoa are closely associated with methanogenesis, since they serve as symbiotic host for methanogens and provide H_2.[11,12] Moreover, ruminal protozoa possess considerable fibrolytic activity associated with higher H_2 production, which ultimately leads to higher methane production.[12] This was supported by the observation of lowered methane production after defaunation of the rumen.[13] Furthermore,

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several studies have evaluated the impact of secondary plant metabolites on rumen methanogenesis. Focusing on tannin- and saponin-rich plant extracts, variable effects such as reduced methane production, increased total gas production, and inhibited growth of protozoa during rumen fermentation were observed.[14−17] However, knowledge on methane mitigation by secondary plant metabolites is still scarce, and reduction strategies should also be technically and economically feasible.

Therefore, the first objective of the present study was to determine the nutritive value of selected fruit processing byproducts. Second, the methane-inhibiting potential of natural feed additives derived from fruit byproducts should be tested in vitro. Aiming at both waste valorization and reduction of methane emissions, byproducts such as mango peels and derived biologically active extracts thereof as well as byproducts of the citrus and apple juice industry should be evaluated with regard to their energy value. In particular, low- (LE) and high-esterified (HE) citrus and apple pectins as well as integral and depectinized mango peels, a phenolic mango peel extract, and a sample containing purified gallic acid were investigated regarding their suitability as feed supplements. The latter represented the predominant bioactive compound of mango peels as shown by LC−MS in this study. The different degrees of esterification of the pectins were evaluated according to their potential impact on methane production, because Keppler et al.[18] also indicated an association of methoxyl groups of pectin and methane formation. Apart from assessing the antimethanogenic effect of these potential feed supplements at different dosages, their influence on total gas production, content of metabolizable energy (ME), and protozoa counts were also determined. In addition, volatile fatty acid (VFA) analysis was carried out in order to provide a more detailed picture of the physiological impact when feeding the above-mentioned byproducts. Acetic and butyric acid are supposed to favor methane production, whereas the production of propionic acid is supposed to use hydrogen in a competitive pathway.[9] In summary, this study provides a comprehensive in vitro assessment of the potential of food processing byproducts for their use as feed supplements, including observations on ruminal methane production.

MATERIALS AND METHODS

Raw Material and Chemicals. Dried mango peels (*Mangifera indica* L. cv. Kaew) were obtained from a research orchard in Chang Mai, Thailand, and stored in the dark at room temperature in double sealed plastic bags until used. After peeling and subsequent frozen storage at −20 °C, mango peels were dried by hot air (80 °C, 4 h) in a TG-1 fluidized-bed dryer (Retsch, Haan, Germany), vacuum sealed, and shipped to Germany at ambient temperature. Commercially available HE and LE apple and citrus pectins were donated by Herbstreith & Fox KG (Neuenbürg, Germany). Other chemicals were purchased from Sigma (St. Louis, MO, USA). Deionized water was used throughout.

Production of Depectinized Peels and the Phenolic Extract from Mango Peels. Pectin extraction was conducted as reported previously.[19] Extraction of phenolic compounds was accomplished as described previously with some modifications.[20,21] After the peels were minced with a laboratory blender, an aliquot of 2 g was extracted with 200 mL of 80% aqueous methanol by continuous stirring at room temperature for 1 h under nitrogen atmosphere to prevent oxidation. Subsequently, the extract was centrifuged (10 min, 2685*g*). After decantation, the solid residues were re-extracted with 200 mL of 80% aqueous methanol for 1 h. The combined methanolic supernatants were evaporated to dryness in vacuo at 35 °C. The dried residue represented the phenolic mango extract used for the gas test described below.

Characterization of Pectin Samples. After removal of acid-soluble constituents, the pectin supplements were further characterized regarding their degree of esterification (DE), methyl ester (MeE) and galacturonic acid (GalA) contents, and degree of acetylation (DA) according to FAO/JECFA methods.[22]

Determination of Phenolic Compounds from Mango Peel Extract. For the chemical characterization of phenolic compounds by HPLC−MS, the above-mentioned phenolic extract was dissolved in 2 mL of methanol and made up to 50 mL with deionized water. The solution was adjusted to pH 2 with 6 M HCl and the aqueous solution was extracted with 50 mL of ethyl acetate. The aqueous phase was separated using a separatory funnel and four times re-extracted with ethyl acetate. The combined organic phases were evaporated in vacuo. The dried residue obtained was dissolved in 1 mL of methanol and 2 mL of deionized water, membrane-filtered (0.45 μm) into an HPLC-vial, and used for HPLC−MS analyses.

The separation of phenolic compounds was performed using a series 1100 HPLC (Agilent, Waldbronn, Germany). Compound identification was carried out by coupling the HPLC system to an Esquire 3000+ ion trap mass spectrometer fitted with an electrospray ionization (ESI) source (Bruker Daltonik, Bremen, Germany). The column used was a 150 mm × 3.0 mm i.d., 4 μm, Synergi Hydro-RP C18, with a 4.0 mm × 2.0 mm i.d. C18 ODS guard column (Phenomenex, Torrance, CA, USA) operated at a temperature of 25 °C. The mobile phase consisted of 1% formic acid in water (solvent A), and of 0.1% formic acid in water/methanol (30/70, v/v; solvent B). The gradient program was as follows: 0% B to 30% B (15 min), 30% B to 40% B (15 min), 40% B to 55% B (15 min), 55% B to 70% B (5 min), 70% B to 100% B (1.5 min), 100% B isocratic (1.5 min), 100% B to 0% B (0.5 min), and re-equilibration of the column at 0% B (11.5 min). Total run time was 65 min at a flow rate of 0.4 mL/min. The injection volume was 5 μL. Phenolic compounds were monitored at 280 and 320 nm. Data acquisition and processing were performed using Esquire Control software. Negative ion mass spectra were recorded in the range m/z 80−1,250 at a scan speed of 13,000 m/z per second. Nitrogen was used both as drying gas at a flow rate of 9.0 L/min and as nebulizing gas at a pressure of 40.0 psi. The nebulizer temperature was set at 365 °C, and a potential of 4,000 V was used on the capillary. For MS^n spectra, helium was used as collision gas for collision-induced dissociation (CID) at a pressure of 4.0×10^{-6} mbar. Isolation width was 6 m/z at a fragmentation amplitude of 1.00 V. Phenolic compounds were identified by their UV/vis absorption and mass spectrometric behavior as published previously.[23−26]

Determination of total phenolics was performed using the Folin−Ciocalteu assay.[27] Gallic acid was used as reference standard.

Hohenheim Gas Test. An in vitro rumen incubation (Hohenheim gas test) was conducted to determine total gas production and methanogenesis.[28,29] The rumen liquor was obtained from two rumen-fistulated, nonlactating Holstein cows prior to morning feeding. Grass hay was given ad libitum, and 2 kg of a dairy concentrate was fed in two equal meals at 8 and 17 h. The rumen fluid was filtered through a multilayered cheesecloth and mixed with a buffer medium (1:2; v/v).[29] An aliquot of 30 mL of the inoculum was filled into prewarmed piston pipettes containing only forage (control) and into piston pipettes with one of the supplements under continuous stirring and CO_2-flushing. The piston pipettes were placed in an incubator (WTB Binder, Tuttlingen, Germany) for 24 h at 39 °C, since adequate observation time for accurate determination of gas production of fermented hay was reported to be 24 h.[30] The buffer used ensured a constant pH of 6.7−6.9 during fermentation of all substrates.

120 mg of hay together with a specified amount of supplement was filled into the incubation vessels, whereby level I represented the lowest and level IV the highest supplement dosage. Additionally, samples solely consisting of hay (control) and supplement, respectively, were analyzed. The treatments are indicated in Table 1. The pectin applications were repeated twice using three incubation vessels for each level (n = 6), whereas the integral and depectinized mango peels, the phenolic extract, and gallic acid were determined

Table 1. Supplementation Levels and Composition of Diets Tested Using the Hohenheim Gas Test[a]

	[mg fresh weight]				
suppl	suppl level I	suppl level II	suppl level III	suppl level IV	suppl only
AP HE	10	20	40		120
AP LE	10	20	40		120
CP HE	10	20	40		120
CP LE	10	20	40		120
mango peels	10	20	40		120
dep MP	10	20	40		120
phenolic extract	5	10	15	20	
gallic acid	5	10	15	20	
hay	120	120	120	120	

[a]AP, apple pectin; CP, citrus pectin; HE, high-esterified; LE, low-esterified; dep MP, depectinized mango peels.

thrice with three repetitions at each level of the respective supplement ($n = 9$). In total, hay (control) plus eight different supplements at four dosage levels were investigated.

The gas volumes resulting after 24 h from fermentation of substrates were calculated by subtracting gas volume of a blank only containing the inoculum.

Methane production was determined after removal of the inoculum by injecting the gaseous phase into an infrared methane analyzer (Pronova Analysentechnik, Berlin, Germany) calibrated with a reference gas (18.0 vol % CH_4) (Air Liquide Deutschland, Düsseldorf, Germany).

For VFA analysis ($n = 6$), Hohenheim gas test was conducted as described above with the following modification. Rumen liquor was obtained from a rumen-fistulated, nonlactating Holstein and Jersey cow prior to morning feeding, respectively, and was mixed subsequently.

Determination of Protozoa. For protozoa count, 1 mL of the rumen fluid after incubation was mixed with 10 mL of a fixation solution consisting of 100 mL of formalin (>37%), 0.6 g of ethyl green (4-[[4-(dimethylamino)phenyl][4-(dimethyliminio)cyclohexa-2,5-dien-1-ylidene]methyl]-*N*-ethyl-*N*,*N*-dimethylanilinium bromide chloride), 8.0 g of NaCl, and 900 mL of deionized water.[31] Protozoa were counted using a Fuchs−Rosenthal microscope slide with integrated counting chamber (0.0625 mm^2, depth: 0.2 mm). Protozoa counts were performed in samples of highest dosage levels (at level III for HE and LE apple and citrus pectin and integral and depectinized mango peels, and at level IV for the phenolic extract and gallic acid) according to Table 1. Additionally, protozoa counts were monitored in all solely incubated samples. Protozoal numbers were counted in three samples of two gas tests, respectively. Counting of each sample was performed in triplicate. Results for HE and LE apple and citrus pectins are tentative, since samples of only one gas test were evaluated.

Analysis of Selected Carbohydrate Fractions. Detergent analysis including neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) was performed in integral and depectinized mango peels as well as hay, respectively, according to VDLUFA[29] methods 6.5.1, 6.5.2, and 6.5.3 based on Van Soest et al.[32] NDF, assayed with a heat stable amylase, and ADF were expressed inclusive of residual ash. Starch and sugar (expressed as sucrose equivalents) were determined in integral and depectinized mango peels, respectively, according to VDLUFA methods 7.2.1 and 7.1.1.[29]

Determination of Further Chemical and Nutritional Characteristics. Digestibility of organic matter (dOM) was calculated using eq 41f, and metabolizable energy (ME) was calculated according to eq 14f.[33] Dry matter (DM), ash, lipid content, and crude protein were analyzed according to the VDLUFA methods 3.1, 8.1, 5.1.1, and 4.1.1 (total *N* multiplied by 6.25), respectively.[29]

Analysis of Volatile Fatty Acids (VFA). Concentrations of VFA were analyzed according to Hildebrand et al.[34] Briefly, after fermentation, an aliquot of 5 mL of rumen liquor was withdrawn from incubation vessels under agitation and centrifuged. Subsequently, 1 mL of the supernatant was mixed with 2-methylvaleric acid in 50% formic acid as internal standard. The samples were frozen and sublimated in vacuo, and the distillate was used for VFA analysis by an HP 6890 Plus gas chromatograph (Agilent, Waldbronn, Germany) equipped with a flame ionization detector and an autosampler HP 7683. Separation was performed using an HP 19091F-112 column (25 m × 0.32 mm × 0.5 μm) (Agilent, Waldbronn, Germany) using helium (purity 5.0) as carrier gas at a constant flow of 1.5 mL/min. Oven temperature was set at 80 °C for 1 min, raised to 205 °C at a rate of 20 °C/min, and held at 205 °C for 2 min. Injector and detector temperature was set to 260 °C each. Injection volume was 0.1 μL, and a split ratio of 40:1 was used. Identification was carried out using authentic standards. Linear calibration curves were generated for quantitation. Data analysis was carried out using ChemStation software.

Statistical Analyses. The data of the in vitro analyses were statistically examined using the general linear model (GLM) procedure of SPSS 20. Differences between days were analyzed using independent samples *t* test for substrates fermented on two different days and one-way ANOVA for substrates fermented on three different days. Means of samples with significant interday variations were tested for significant differences using $n = 2$ and $n = 3$ (1 day = 1 replicate). In contrast, means of samples without interday variations were compared using $n = 6$ and $n = 9$ (1 fermentation = 1 replicate), respectively. For evaluation of the effect of different fermentation substrates on total gas and methane production, GLM univariate ANOVA procedures were used, and statistically significant differences between means were identified by the Duncan's test. Differences were considered significant at $p < 0.05$. Results are given in mean ± standard deviation.

RESULTS AND DISCUSSION

Characterization of Proposed Feed Supplements. Integral and depectinized mango peels as well as the pectins were relatively low in ash, crude protein, and lipids (Table 2). As illustrated in Table 3, content of detergent fiber was higher in depectinized mango peel as compared to integral mango peel. NDF of integral and depectinized mango peel was 16.9%

Table 2. Chemical Composition, Digestibility of Organic Matter, and Metabolizable Energy of Feed Supplements[a]

	DM [%]	ash [% in DM]	crude protein [% in DM]	lipids [% in DM]	dOM [%]	ME [MJ/kg DM]
MP	96.06	2.79	3.66	0.88	80.1	11.92
dep MP	61.08[b]	1.95	6.60	2.63	76.6	11.85
AP HE	89.32	5.85	1.60	0.56	84.9	12.21
AP LE	88.78	6.29	1.48	0.35	86.5	12.35
CP HE	90.32	1.46	2.50	0.09	85.9	12.83
CP LE	90.17	5.42	3.79	0.28	84.7	12.16

[a]DM, dry matter; dOM, digestibility of organic matter; ME, metabolizable energy; MP, mango peels; dep MP, depectinized mango peels; AP, apple pectin; CP, citrus pectin; HE, high-esterified; LE, low-esterified. [b]For VFA analysis, dry matter of dep MP was 90.0%.

Table 3. Detergent Fiber, Starch, and Sugar Content of Selected Feed Supplements[a]

	[% in DM]				
	NDF	ADF	ADL	starch	sugar[b]
hay	52.01	25.15	2.19		
MP	16.86	11.70	4.26	7.67	37.81
dep MP	36.40	33.53	10.86	19.63	13.40

[a]MP, mango peels; dep MP, depectinized MP; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin. [b]Determined as sucrose equivalents.

and 36.4% in DM, respectively, whereas hay showed the highest NDF content (52.0% in DM). ADF and ADL were highest for depectinized mango peel with 33.5% and 10.9% in DM, respectively. ADF and ADL of integral mango peel was 11.7% and 4.3% and for hay 25.2% and 2.2% in DM, respectively. Sugar content was considerably higher in mango peels (37.8% in DM) as compared to depectinized peels (13.4% in DM), whereas pectin extraction led to accumulated starch content in depectinized peels (19.6% in DM) in contrast to integral peels (7.7% in DM). Integral mango peels might be considered a promising feed source, since their dOM (80.1%) and ME (11.9 MJ/kg DM) (Table 2) come close to barley and sugar beet pulp having a dOM of 85% and 86% and ME of 12.8 and 11.9 MJ/kg DM, respectively.[35] Nutritional properties of mango peels are also comparable to apple pomace with ME of 11.4 MJ/kg DM.[36] In contrast to depectinized apple pomace (ME 6.9 MJ/kg DM) studied by Steingass and Haussner,[36] depectinized mango peel still was revealed to potentially be a promising feed source due to its high ME amounting to 11.8 MJ/kg DM. As shown in Table 3, high amounts of starch (8−20%) and sugar (13−38%) contributed to the determined high ME of integral and depectinized mango peels.

As indicated in Table 4 and the related chromatogram (Figure 1), characterization and quantitation of phenolic compounds in a mango peel extract revealed gallic acid as the major phenolic compound, due to identification of free gallic acid (compound **1**), its glycosylated derivatives (compounds **2**, **4**, **6**, **7**, **9**, **10**, **14**−**17**), gallic acid polymers (compounds **3**, **8**, **11**), and other derivatives (compounds **5**, **12**, **13**). In conclusion, gallic acid derivatives dominated the phenolic profile of the methanolic extract from integral mango peels. In agreement with previous reports,[37] total phenol content of the methanolic extract of mango peel amounted to 56.3 ± 0.3 mg gallic acid equivalents/g DM. Representing a rich source of polyphenols, mango peels contain >5-fold total phenolics compared to dried apple pomace (10.2 mg gallic acid equivalents/g DM).[38]

Table 5 shows different specifications of the HE and LE apple and citrus pectin, respectively. Since methanogenesis was affected by methoxyl groups of pectins,[18] methyl ester content (MeE) was of particular interest, raising expectations of lower methane production after supplementing LE pectins. As specified by the pectin supplier, MeE was about 8−9% and ∼5% in the HE and LE samples, respectively. In accordance

Table 4. UV and Mass Spectral Characteristics of Gallic Acid and Its Derivatives from a Mango Peel Extract

peak no.	identity	t_R[a] [min]	UV/vis abs max [nm]	$[M - H]^-$ m/z	HPLC−ESI(−)-MS^n m/z (% base peak)
1	gallic acid	8.2	235, 273	169	MS^2 [169]: 125 (100)
2	monogalloyl glucose	10.4	236, 274, 297	331	MS^2 [331]: 271 (100), 169 (14)
					MS^3 [331 → 271]: 211 (100), 169 (31), 125 (8)
3	digallic acid	16.4	233, 281	321	MS^2 [321]: 169 (100), 125 (11)
					MS^3 [321 → 169]: 125 (100)
4	maclurin-*O*-galloyl glucose	17.3	235, 280, 323 sh	575	MS^2 [575]: 303 (100), 285 (49), 193 (30), 313 (25), 423 (24)
					MS^3 [575 → 303]: 193 (100), 167 (71), 165 (56), 105 (10)
5	methyl gallate	18.5	232, 272	183	MS^2 [183]: 168 (100), 124 (8)
					MS^3 [183 → 168]: 124 (100)
6	digalloyl glucose	19.3	233, 282	483	MS^2 [321]: 169 (100), 125 (11)
					MS^3 [321 → 169]: 125 (100)
7	maclurin-di-*O*-galloyl glucose	20.1	234, 280, 324 sh	727	MS^2 [727]: 575 (100), 465 (39), 485 (14)
					MS^3 [727 → 575]: 485 (100), 269 (72), 285 (66), 405 (66)
8	digallic acid	21.0	232, 273	321	MS^2 [321]: 169 (100), 125 (11)
					MS^3 [321 → 169]: 125 (100)
9	tetra-*O*-galloyl	33.0	232, 278	787	MS^2 [787]: 635 (100), 617 (34)
10	glucose	33.4			MS^3 [787 → 635]: 483 (100), 465 (85), 313 (64), 253 (54), 423 (49)
11	trigallic acid	35.6	231, 273	473	MS^2 [473]: 321 (100), 169 (32)
					MS^3 [473 → 321]: 169 (100), 125 (7)
12	mangiferin gallate	37.2	232, 261 sh, 276	573	MS^2 [573]: 421 (100), 301 (24), 331 (21), 403 (13)
					MS^3 [573 → 421]: 301 (100), 331 (38)
13	isomangiferin gallate	39.2	231, 259 sh, 277	573	MS^2 [573]: 421 (100), 283 (33), 331 (19), 403 (19), 301 (13)
					MS^3 [573 → 421]: 301 (100), 331 (15), 302 (15), 273 (13)
14	penta-*O*-galloyl glucose	41.4	232, 281	939	MS^2 [939]: 769 (100), 770 (31), 617 (26), 787 (13)
					MS^3 [939 → 769]: 617 (100), 601 (23), 295 (14), 447 (13), 618 (13)
15	hexa-*O*-galloyl	45.2	231, 280	1091	MS^2 [1091]: 939 (100), 769 (24)
16	glucose	46.8			MS^3 [1091 → 939]: 769 (100), 617 (27), 770 (11)
17	hepta-*O*-galloyl glucose	49.1	231, 277	1243	MS^2 [1243]: 939 (100), 1091 (34), 769 (16)
					MS^3 [1243 → 939]: 769 (100)

[a]Retention time.

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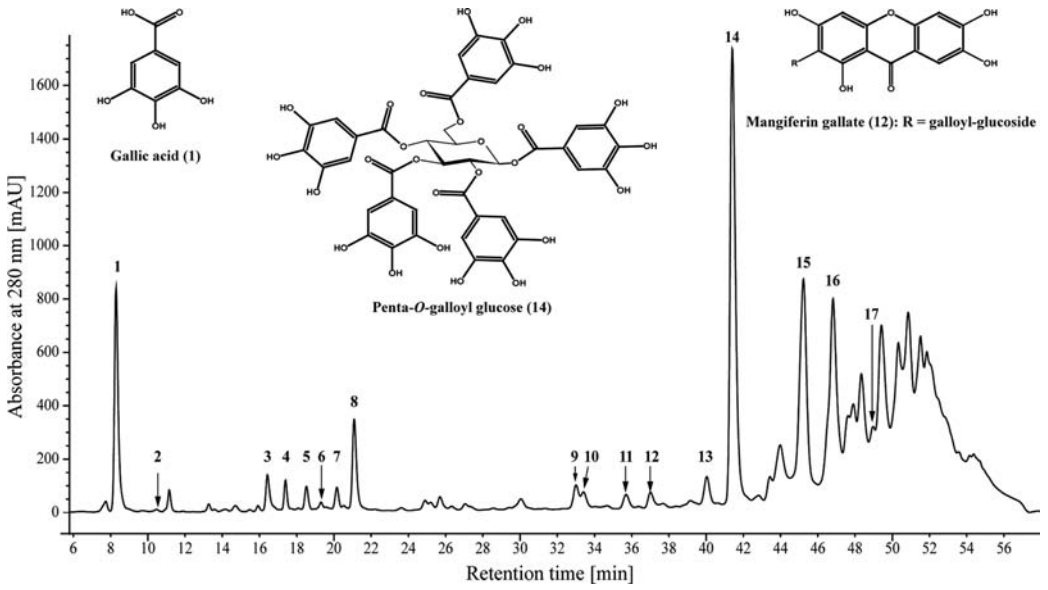

Figure 1. Separation of phenolic compounds from mango peel by HPLC monitored at 280 nm. For peak assignment see Table 5.

Table 5. Chemical Characterization of Pectin Samples[a]

	DE [%]	GalA [%]	MeE [%]	DA [%]
AP HE	68.0	73.9	8.0	2.8
AP LE	38.2	81.0	4.9	1.7
CP HE	70.3	77.1	8.7	4.1
CP LE	34.7	82.6	4.6	2.5

[a]DE, degree of esterification; GalA, galacturonic acid content; MeE, methyl ester content; DA, degree of acetylation; AP, apple pectin; CP, citrus pectin; HE, high-esterified; LE, low-esterified.

with these findings, the HE pectins had a degree of esterification (DE) of >68%, whereas DE of the LE pectins was <40%. Galacturonic acid contents in the LE samples ranged from 73.9% (apple) to 77.1% (citrus), being slightly higher for the HE pectins. The degree of acetylation (DA) was within the range of 1.7−4.1% for all samples, with higher DAs for HE pectins (Table 5). Moreover, apple and citrus pectins showed the highest energy content of the studied substrates with ME > 12 MJ/kg DM and dOM of 84.7−86.5%.

In summary, integral and depectinized mango peels as well as apple and citrus pectins possess a considerable amount of digestible carbohydrates, making them well suited as putative energy supplements in ruminant rations. For exhaustive exploitation and optimal valorization of integral mango peel, the residual material resulting from pectin extraction may be used as a feed supplement, while pectin may be mainly destined for food applications.

Effects of Proposed Feed Supplements on in Vitro Fermentation. Since ruminal methanogenesis substantially contributes to global warming by promoting the greenhouse gas effect, multiple studies aiming at implementation of antimethanogenic strategies such as defaunation of the rumen have been conducted.[13,17] To determine the influence of different substrates on digestion and methanogenesis using the in vitro gas test, both total gas and methane production were measured, since gas production closely reflects their digestibility.[33]

Gas yield after fermentation of the control (hay) totaled to 24.8 ± 0.7 mL/100 mg DM. When substituting the hay by mango peel and the different pectins, a significantly higher total gas production was observed (Figure 2A) ($p < 0.05$). Yielding up to 38.6 mL/100 mg DM for HE citrus pectin, the pectin samples generally revealed greatest total gas production. Considering the same substitution experiment, total gas yield after fermentation of depectinized mango peels (32.2 ± 1.6 mL/100 mg DM) was only slightly lower than for integral peels (34.6 ± 1.6 mL/100 mg DM). Higher total gas volumes may have been generated after integral peel fermentation, possibly due to its high sugar and starch content of 37.8% and 7.7%, respectively (Table 3).

Besides replacement of hay, the basic hay diet was supplemented with the integral and depectinized peel, the phenolic peel extract, gallic acid, and the four pectins at up to four different dosage levels, respectively. Considering these supplementation experiments, a significantly diminished total gas production was obtained by the addition of the phenolic extract at levels I and II as compared to the control (hay only) (Figure 2B). Irrespective of the level of the supplementation, gallic acid induced a significant decrease in gas production ($p < 0.05$), except for level III supplementation. Since total gas production was decreased for gallic acid supplementation, lower gas production might analogously be attributed to the presence of gallic acid and its derivatives in the phenolic extract. In accordance with our findings, similar effects of polyphenols like condensed tannins on rumen fermentation have been described previously.[14] Unexpectedly, supplementation of peels and pectin to hay diets resulted in equal and for depectinized mango peel in one case in even lower total gas production (Figure 2B,C), although the total substitution of hay by peels and pectin resulted in increased gas production. The reason for this observation remains unknown. Effects related to pH changes were excluded, as the inoculum was strongly buffered and pH was constantly between 6.7 and 6.9 for all samples, indicating that substrate dosage and VFA production did not affect pH. It might be speculated if the combination of hay and peels or pectins has increased efficiency of microbial biomass synthesis on the cost of the fermentation end products CO_2, methane, and VFA as postulated by Blümmel et al.[39] By analogy to total gas production, methanogenesis was influenced by substituting and supplementing the hay-based diets.

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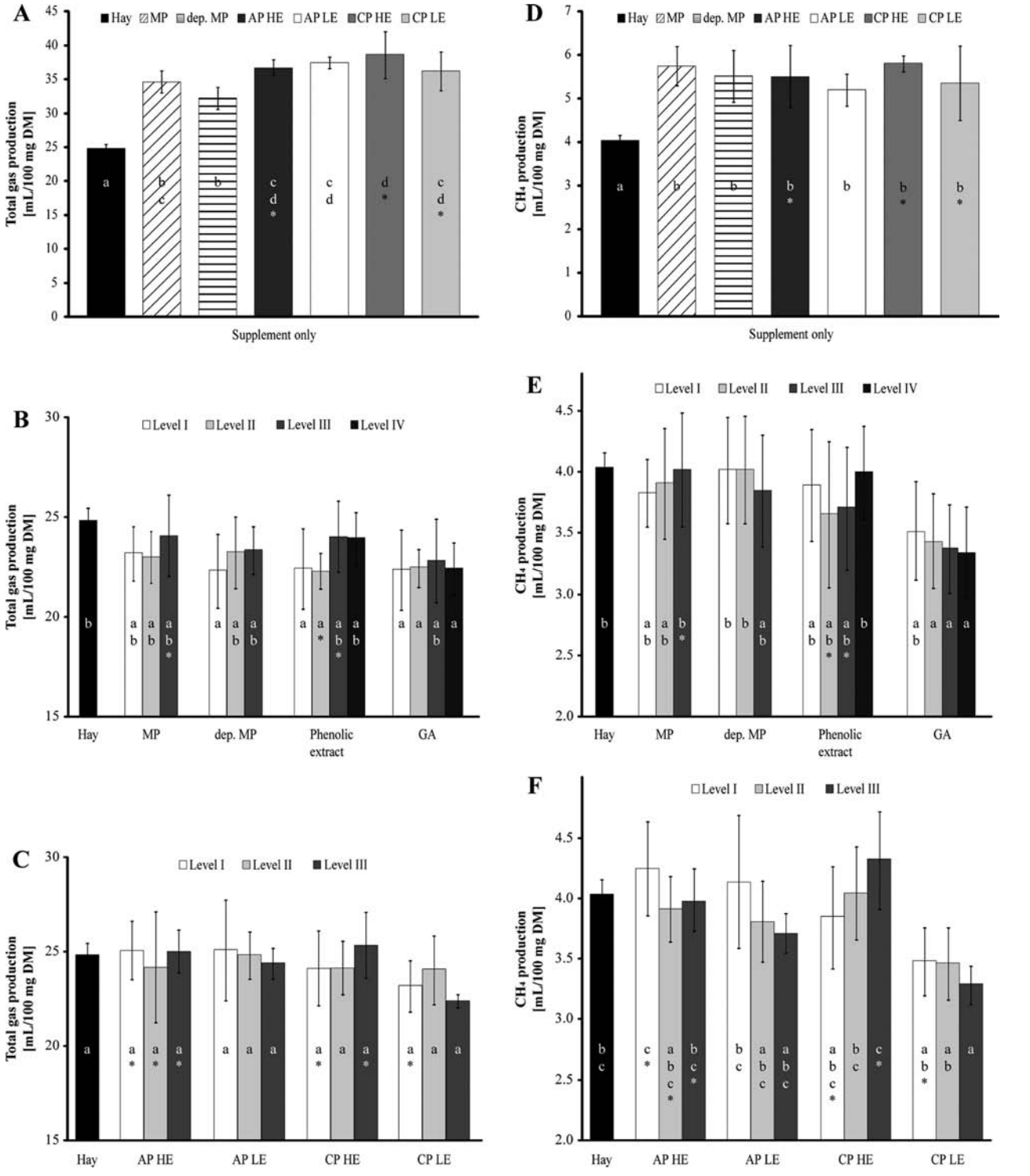

Figure 2. Total gas and methane production after in vitro digestion of analyzed supplements: [A–C] total gas production; [D–F] methane production; [A and D] substitution of hay by mango peels (MP), depectinized MP (dep. MP), and high- (HE) and low-esterified (LE) apple (AP) and citrus pectin (CP) only; [B and E] supplementation of hay with MP, depectinized MP (dep. MP), phenolic extract, and gallic acid (GA); [C and F] supplementation of hay with high- (HE) and low-esterified (LE) apple (AP) and citrus pectin (CP). Statistical analysis for results marked with "*" was based on $n = 2$ (corresponding pectin samples) and $n = 3$ (corresponding mango peel and phenolic extract samples) due to significant interday differences. $n = 6$ and $n = 9$ was used for samples without interday differences, respectively.

Methane production was increased by 28–43% when hay (4.0 ± 0.1 mL/100 mg DM) was substituted (Figure 2D). When hay was replaced by integral and depectinized mango peels, methane production significantly increased to 5.8 ± 0.5 and 5.5 ± 0.6 mL/100 mg DM, respectively. As expected, fermentation of HE pectin (apple, 5.5; citrus, 5.8 mL/100 mg DM) resulted in higher methane yields than LE pectins (apple, 5.2; citrus, 5.3 mL/100 mg DM), since additional methyl esters of galacturonic acid may be cleaved from HE pectins. Methanol generated by de-esterification of pectins may be metabolized by

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Table 6. Volatile Fatty Acid Production and Corresponding Gas Production after 24 h in Vitro Fermentation of Substrates[a]

		supplement only							level III		level IV			level III			
	hay[b] only	MP	dep MP	AP HE	AP LE	CP HE	CP LE	hay[b] only	MP	dep MP	phe extr	GA	hay[b] only	AP HE	AP LE	CP HE	CP LE
volatile fatty acids																	
[μmol/100 mg DM]																	
C2	323 a	469 c	376 b	573 d	587 d	646 e	625 e	323 b	348 c	339 bc	330 bc	284 a	323 a	378 b	378 b	390 b	392 b
C3	114 c	157 e	125 d	103 b	94 a	98 ab	97 a	114 b	121 c	117 bc	112 b	94 a	114 a	113 a	110 a	111 a	113 a
C4	33 d	54 e	29 c	20 b	18 a	19 ab	19 ab	33 b	39 c	34 b	32 ab	30 a	33 b	30 a	29 a	30 a	30 a
C5	3 d	4 e	3 bc	2 a	2 a	3 cd	3 b	3 b	3 b	3 b	3 b	3 a	3 b	3 a	3 a	3 b	3 b
total	475 a	684 c	533 b	700 c	702 c	768 d	745 d	475 b	511 c	494 bc	478 b	412 a	475 a	525 b	522 b	535 b	539 b
[%]																	
C2	68.11 a	68.55 b	70.55 c	81.88 d	83.55 e	84.08 e	83.83 e	68.11 a	68.03 a	68.66 ab	69.10 b	69.03 b	68.11 a	72.08 b	72.44 bc	72.84 c	72.61 bc
C3	23.98 e	22.93 d	23.41 de	14.79 c	13.44 b	12.76 a	12.98 ab	23.98 b	23.63 b	23.62 b	23.35 ab	22.73 a	23.98 c	21.48 b	21.12 ab	20.76 a	20.87 a
C4	6.95 d	7.95 e	5.41 c	2.88 b	2.58 a	2.51 a	2.58 a	6.95 c	7.54 d	6.81 b	6.64 a	7.29 c	6.95 b	5.63 a	5.62 a	5.54 a	5.63 a
C5	0.70 d	0.54 c	0.57 c	0.34 a	0.31 a	0.42 b	0.39 b	0.70 b	0.64 a	0.68 b	0.71 b	0.71 b	0.70 b	0.59 a	0.59 a	0.61 a	0.63 a
GP [mL/100 mg DM]	23.64	36.52	29.22	40.96	40.06	43.69	40.05	23.64	26.65	24.25	24.72	22.13	23.64	28.81	27.88	29.71	27.61
CH_4 [mL/100 mg DM]	5.48	9.01	6.31	7.72	6.61	8.78	7.53	5.48	5.49	5.24	4.44	3.86	5.48	6.35	6.17	6.69	4.95

[a]GP, total gas production; CH_4, methane production; C2, acetic acid; C3, propionic acid; C4, butyric acid; C5, valeric acid; DM, dry matter; MP, mango peels; dep MP, depectinized mango peels; AP, apple pectin; CP, citrus pectin; HE, high-esterified; LE, low-esterified; phe extr, phenolic extract; GA, gallic acid. Supplement only, level III, and level IV according to Table 1. [b]The same hay results for statistical assessment of each group were used.

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methanogens in methanogenic pathways.[40] In agreement with this hypothesis, the highest methane production was observed when substituting hay with HE citrus pectin (5.8 mL/100 mg DM) characterized by the highest degree of esterification (70.3%) (Table 5).

When the hay-based diet was supplemented with one of the pectins, methane production was either decreased by up to 19% (LE citrus pectin, level III) or increased by up to 7% using HE citrus pectin (level III). Purified gallic acid was revealed to be an efficient additive to suppress methane production by up to 17% (Figure 2E). Since total phenol content of mango peels amounted to 56.3 ± 0.3 mg gallic acid equivalents/g DM, they represent a rich source of polyphenols, in particular gallic acid (Figure 1). High levels of mango peel-derived gallic acids were even enriched in the phenolic mango peel extract, where these compounds and other major phenolic constituents of mango peels like mangiferin and quercetin, as shown by Berardini et al.,[41] might represent an inhibitory principle of methanogenesis. By trend, a reduction of methane emission of 9% was observed with the addition of the phenolic mango peel extract (level II, Figure 2E). Unlike the gallic acid supplement, the mango extract contained further water- and methanol-soluble constituents such as mono- and oligosaccharides, which might increase methane production. The antagonistic action of antimethanogenic phenolics and methanogenic nutrients present in the phenolic mango extract may explain the weaker suppression of methanogenesis by the extract as presented in Figure 2E. Besides gallic acid, also LE pectins remarkably diminished methane production (Figure 2F). The lower methane production from LE citrus pectin was in contrast to observations of Pol and Demeyer,[42] who found an increase in methane production from pectins and methanol in vitro when using an adapted rumen inoculum. However, Genthner et al.[43] demonstrated the ability of *Eubacterium limosum* isolated from rumen contents to produce acetic acid from methanol. Poulsen et al.[44] also found lower methane production from pectin fermentation with rumen fluid in vitro as compared to substrates rich in starch, especially in the first 10 h of incubation. Nevertheless, supplementation of hay with LE apple and citrus pectins yielded lower methane emissions (3.3−4.1 mL/100 mg DM) than their HE counterparts (3.9−4.3 mL/100 mg DM). For LE citrus pectin, having the lowest degree of esterification (34.7%), methane reduction was most intense (3.3 mL/100 mg DM, Figure 2F). As shown in Table 6, total gas and methane production of the VFA experiment showed similar results as the main analysis for these parameters (Figure 2), with increased total gas and methane production in the substitution experiment as compared to hay. The repeated experiment confirmed the trend of attenuated methanogenesis by supplementation of LE citrus pectin and gallic acid.

Combining the results of total gas and methane production, an ideal supplement should suppress methanogenesis, however, without impairing total gas production, since the latter is positively correlated with the digestibility of a substrate.[33] Broudiscou et al.[45] described attenuation of methanogenesis by 8.2% and 14.2% when supplementing diets of ruminants with extracts of *Salvia officinalis* L. and *Equisetum arvense* L., respectively. Unfortunately, total gas production was also remarkably depressed at the same time. In our study, the gallic acid supplement was found to be an efficient suppressor of methanogenesis. However, it also lowered total gas production and, thus, might affect the nutritional value of the feed. In contrast, feed supplementation with LE citrus pectin yielded outstandingly low methane emissions without affecting total gas production. Therefore, LE citrus pectin may represent a promising feed additive for the mitigation of methane emission by ruminants.

Effects of Proposed Feed Supplements on Protozoa. Both substitution and supplementation of the hay-based diets consistently increased protozoal numbers (Figure 3). Irrespective of the additive applied for substituting hay, both methane emissions and protozoa counts rose (Figure 3, dashed circle). As in the case of methanogenesis, higher contents of starch and sugar of the substitutes may also enhance protozoa propagation. By analogy, the supplementation of pectin to hay resulted in higher numbers of protozoa. Considering pectin supplemented diets, a positive correlation between protozoa counts and methane production was observed (R^2 = 0.99), as illustrated by the gray area in Figure 3. Therefore, our study indicates a simultaneous influence of pectin on protozoa and, as hypothesized above, methanogenic microorganisms. In good agreement, Morgavi et al.[12] previously reported a close association of methanogens and protozoa. Compared to the pectin supplementation, protozoal numbers were lower when the phenolic extract was supplemented. At the same time, methane production did not differ remarkably as compared to the control and the pectins. Comparing the control with gallic acid and LE citrus pectin supplementation experiments, an increased protozoal number was associated with a lowered methane production. The decreased methane release may be explained by the inhibitory effects of gallic acid and LE citrus pectin on methanogens. In contrast, protozoa remained widely unaffected by these supplements. LE citrus pectin may even be metabolized by protozoa, and therefore supported protozoal growth. Since Hess et al.[46] found decreased numbers of ciliate protozoa when studying the effect of diets supplemented with the tannin-rich *Sapindus saponaria* L. fruits, the increased protozoa count following gallic acid supplementation was unexpected. When supplementing diets for wethers with extracts of *Yucca schidigera* Roezl ex Ortgies and *Quillaja saponaria* Molina, respectively, protozoa counts were also found

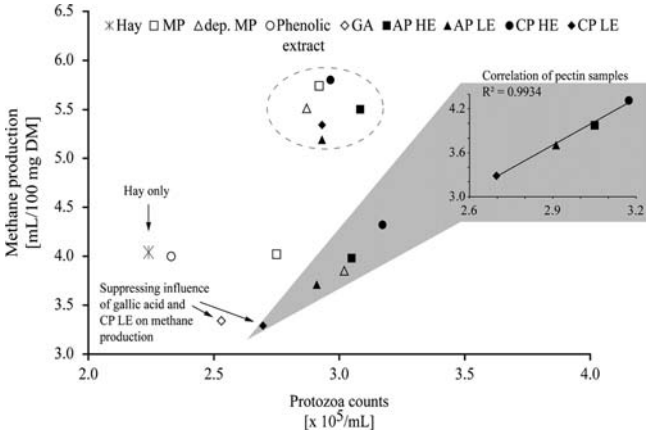

Figure 3. Protozoa per mL after fermentation for 24 h in relation to methane production. Results are given for supplements only (dashed circle) and level III or IV (highest level of the corresponding supplement according to Table 1). Gray area highlights the correlation of protozoal numbers and methane production when different types of pectin were used. MP: mango peels. Dep MP: depectinized mango peels. GA: gallic acid. AP: apple pectin. CP: citrus pectin. HE: high-esterified. LE: low-esterified.

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to decline.[47] Possibly, our findings may be explained by a specific metabolization of gallic acid, since microbial decarboxylation and dehydroxylation of phenolic acids and metabolization of gallates have been previously reported.[48,49]

Effects of Proposed Feed Supplements on VFA Production. Analysis of VFAs revealed a major production of acetic (C2), propionic (C3), butyric (C4), and valeric acid (C5), whereas only minor amounts of iso forms of butyric and valeric acid were detected (<2 μmol/100 mg DM), which are, therefore, not shown. Total amount and ratio of the individual VFAs after substrate fermentation are shown in Table 6. A positive correlation (R^2 = 0.97) was observed when total VFA production was plotted against total gas production (Figure 4),

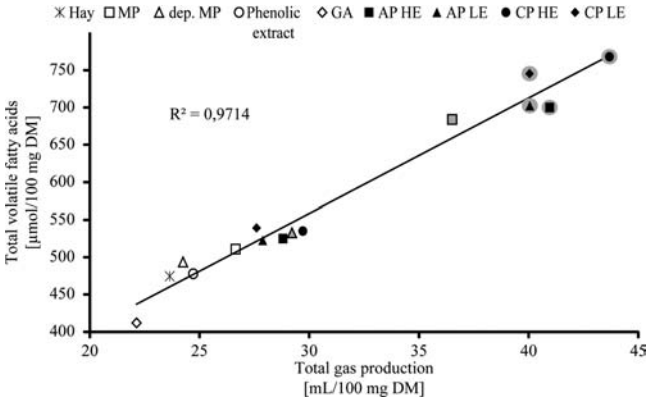

Figure 4. Correlation of total gas production and total volatile fatty acids. Results are given for supplements only (gray background) and level III or IV (highest level of the corresponding supplement according to Table 1). MP: Mango peels. Dep MP: depectinized mango peels. GA: gallic acid. AP: apple pectin. CP: citrus pectin. HE: high-esterified. LE: low-esterified.

irrespective of substrates. Quantitatively, substitution of hay by the substrates consistently resulted in an increased total VFA production (533−768 μmol/100 mg DM) as compared to hay (475 μmol/100 mg DM), as shown in Table 6. Fermentation of integral mango peel (684 μmol/100 mg DM) produced significantly more VFAs than depectinized mango peel (533 μmol/100 mg DM). Moreover, total VFA production increased when pectins (≥700 μmol/100 mg DM) were fermented in vitro.

Qualitatively, a significantly higher proportion of C2 (>81%) was determined for all pectins at the expense of C3 (<15%), C4 (<3%), and C5 (≤0.4%) when compared to hay only (C2, 68%; C3, 24%; C4, 7%; C5, 0.7%). According to Van Soest,[50] bacteria from *Succinivibrio dextrinosolvens*, *Lachnospira multiparus*, *Bacteroides ruminicola*, and *Streptococcus bovis* are the main pectin utilizing microorganisms producing mainly acetate and formate. In accordance with our findings, Poulsen et al.[44] reported high yield of acetate and very low shares of propionate and butyrate when pectins were fermented in vitro. Due to their high acetate producing potential, pectin feeds could be used as supplements for dairy cow diets to counteract possible milk fat depression caused by high propionate production when feeding diets rich in starchy concentrates.

By analogy, during the supplementation experiment total VFA production increased (>520 μmol/100 mg DM) and a shift of C2 (>72%) was determined by supplementation of pectins as compared to hay only (475 μmol/100 mg DM and 68%, respectively). Moreover, pectin supplementation resulted in similar total VFA amounts (522−539 μmol/100 mg DM) and VFA profiles (C2, 72%; C3, 21%; C4, 5%; C4, 0.6%) among themselves. As compared to hay, supplementation of integral and depectinized mango peels also resulted in increased VFA production (>490 μmol/100 mg DM) and similar VFA profiles.

In contrast, supplementation of gallic acid resulted in decreased total VFA production (412 μmol/100 mg DM). Simultaneously, total gas production decreased after gallic acid supplementation (22.1 mL/100 mg DM), whereas increased total gas production was determined for the pectins (>27.6 mL/100 mg DM) as compared to hay (23.6 mL/100 mg DM). However, as compared to hay, reduced methane production after supplementation of gallic acid and LE citrus pectin to hay could be associated neither to individual nor to total VFA production.

In summary, the present study evaluated the suitability of fruit processing byproducts such as mango byproducts, extracts thereof, and various pectins as valuable feed supplements and, simultaneously, their potential to attenuate rumen methanogenesis. HE and LE citrus and apple pectins as well as integral and depectinized mango peels revealed promising potential for utilization as feed or feed additives due to their presented chemical composition, in vitro digestibility, and favorable VFA composition. Among the supplements tested, a purified gallic acid supplement and LE citrus pectin reduced methanogenesis by 17% and 19%, respectively. While gallic acid significantly suppressed total gas production, LE citrus pectin did not cause undesired reduction of total gas production. However, further in vivo studies are needed. Contributing to sustainability by valorization of fruit processing waste, greenhouse gas emissions caused by ruminants may be reduced at the same time, thus achieving a more efficient feed use.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full table of volatile fatty acids (see Table 6) including standard deviation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ADF, acid detergent fiber; ADL, acid detergent lignin; AP, apple pectin; C2, acetic acid; C3, propionic acid; C4, butyric

acid; C5, valeric acid; CP, citrus pectin; DA, degree of acetylation; DE, degree of esterification; dep. MP, depectinized mango peel; DM, dry matter; dOM, digestibility of organic matter; GA, gallic acid; GalA, galacturonic acid; HE, high-esterified; LE, low-esterified; ME, metabolizable energy; MeE, methyl ester; MP, mango peel; NDF, neutral detergent fiber; VFA, volatile fatty acids

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