https://doi.org/10.17221/118/2017-CJAS

In Vitro Bioactivity of Various Pure Flavonoids in Ruminal Fermentation, with Special Reference to Methane Formation

*Susanne Sinz[1], Carmen Kunz[1], Annette Liesegang[2], Ueli Braun[3], Svenja Marquardt[1], Carla R. Soliva[1†], Michael Kreuzer[1]**

[1]*ETH Zürich, Institute of Agricultural Sciences, Zürich, Switzerland*

[2]*Institute of Animal Nutrition, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland*

[3]*Department of Farm Animals, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland*

**Corresponding author: michael.kreuzer@inw.agrl.ethz.ch*

ABSTRACT

Sinz S., Kunz C., Liesegang A., Braun U., Marquardt S., Soliva C.R., Kreuzer M. (2018): ***In vitro* bioactivity of various pure flavonoids in ruminal fermentation, with special reference to methane formation**. Czech J. Anim. Sci., 63, 293–304.

Polyphenols, like flavonoids, have been investigated when present in intact plants or in extracts as methane mitigating dietary supplements in ruminants. The aim of the present study was to examine pure compounds in a short-term *in vitro* experiment using the Hohenheim Gas Test method. We focused on the group of the flavonoids and tested which of them had the potential to mitigate methane without negatively affecting ruminal fermentation. Eight flavonoids were tested: epicatechin, luteolin-7-glucoside, quercetin, and isoquercetin in Experiment 1; catechin, gallocatechin, epigallocatechin, and epigallocatechin gallate in Experiment 2. Tannic acid, no flavonoid but a phenolic acid with known methane mitigating properties, served as positive control, and the unsupplemented basal diet as negative control. In both experiments, each of these compounds (including tannic acid) was tested at dosages of 0.5, 5.0, and 50.0 mg/g basal diet dry matter (DM) in four runs each. Gallocatechin, tannic acid, and epigallocatechin gallate (50 mg/g DM) lowered fermentation gas formation and *in vitro* organic matter digestibility relative to the negative control (Experiment 2). Apart from tannic acid, epicatechin, quercetin, isoquercetin, and luteolin-7-glucoside (5 and 50 mg/g DM) reduced the amount of CH_4 produced in relation to total gas produced (Experiment 1). The incubation fluid ammonia concentration was decreased with luteolin-7-glucoside and tannic acid (50 mg/g DM). From the flavonoids tested especially luteolin-7-glucoside seems to have a similar potential as tannic acid to mitigate methane and ammonia formation during ruminal fermentation *in vitro*, both favourable in environmental respect. These results need to be confirmed in live animals.

Keywords: rumen; ammonia; Hohenheim Gas Test; epicatechin; isoquercetin; luteolin-7-glucoside; quercetin; catechin; tannic acid

The interest in mitigating greenhouse gases produced in agriculture strongly increased over the last few years. The focus of research has turned towards system optimisation in terms of animal and environmental health as well as animal-source food quality. Most promising in achieving beneficial

Partially supported by the Swiss National Science Foundation (Grant 320030_149976).
The authors declare no conflicts of interest.
†Carla R. Soliva has passed away.

https://doi.org/10.17221/118/2017-CJAS

effects in ruminant nutrition are the representatives of the extensive class of the polyphenols. Such possible effects include methane (CH_4) suppression (Beauchemin et al. 2008; Cieslak et al. 2013), ruminal by-pass of valuable dietary protein (Cortes et al. 2009) and positive effects on animal health, for instance via antioxidant properties (Bjorklund and Chirumbolo 2017). However, polyphenols may also act in an anti-nutritive manner in ruminants, which can be associated with a reduced ruminal organic matter degradation and, thus, energy supply of the animal (McSweeney et al. 2001).

Polyphenols are plant secondary compounds and can be divided in two large groups: the phenolic acids and the flavonoids. Flavonoids are subdivided into six sub-groups: flavanonols, flavonols, flavanons, flavons, anthocyanidins, and isoflavones (Crozier et al. 2006). Oligomeric and polymeric flavanols are called proanthocyanidins or condensed tannins (CT), whereas hydrolysable tannins (HT) are built from the phenolic acids gallotannins and ellagitannins (Crozier et al. 2006). Some, but not all extracts containing tannins (CT or HT or both) were shown to exhibit effects on ruminal fermentation and methanogenesis (Jayanegara et al. 2012). Since CT and HT consist of a number of sub-units, these effects cannot be allocated to the respective compounds like for instance single flavonoids. Therefore, the aim of the present study was to elucidate with pure substances which flavonoid sub-units, some of them being part of polyphenols like CT, are responsible for effects on ruminal fermentation and methane emission.

In the present study, the following hypotheses were tested: (1) There is a number of distinct flavonoids originating from different chemical groups, which are bioactive in rumen fermentation similarly to tannic acid (a phenolic acid with known bioactive properties). (2) At least some of the flavonoids significantly reduce either CH_4 emission or NH_3 formation or both without compromising the general ruminal nutrient fermentation. (3) The effects of the flavonoids are dose dependent. This was tested in two experiments using a common short-term *in vitro* system.

MATERIAL AND METHODS

Test compounds. A total of eight pure flavonoids (five flavanols, two flavonols, and one flavon) were tested, four flavonoids in each of two experiments, in addition to tannic acid (positive control; Figure 1). All compounds were available in pulverized form from Sigma-Aldrich Chemie GmbH, Buchs, Switzerland. In Experiment 1, epicatechin (purity ≥ 90%), a flavanol representative of the sub-units of CT, was included. Additionally, two flavonols, quercetin (purity ≥ 98%) and isoquercetin (quercetin-3-β-glucopyranoside; purity ≥ 90%) (all Sigma-Aldrich) were tested. Isoquercetin is a naturally occurring glucoside of quercetin. Finally, luteolin-7-glucoside (purity ≥ 98%; Sigma-Aldrich), a flavone being a glucosidic form of luteolin, was included. Apart from epicatechin used only in Experiment 1, the four following flavanols (all Sigma-Aldrich) were tested in Experiment 2. Catechin (purity ≥ 96%) is composed of two benzene rings and a dihydropyran heterocycle. Gallocatechin (purity ≥ 97%) and epigallocatechin (purity ≥ 95%) differ only in their configuration. Both have the same chemical structure as catechin except of an additional OH-group on the dihydropyran heterocycle. Epigallocatechin gallate (purity ≥ 92%), an ester of epigallocatechin and gallic acid, comprised the last test substance (Crozier et al. 2006).

For both experiments, tannic acid (Sigma-Aldrich) served as positive control. It belongs to the group of phenolic acids, one of the sub-groups of phenolic compounds and is a subunit present in HT (Crozier et al. 2006). There is ample evidence that HT may mitigate methane in ruminants (Jayanegara et al. 2012), and tannic acid has been demonstrated to possess methane mitigations effects in other *in vitro* studies (Field and Lettinga 1987; Yang et al. 2017). In both experiments, a non-supplemented treatment served as negative control.

In each experiment, in total five different compounds (including tannic acid) were tested at dosages of 0.5, 5.0, and 50.0 mg/g basal diet dry matter (DM). These levels had been successfully used in an *in vitro* experiment using the same experimental set-up and the same negative and positive control when testing the flavonoid rutin (Leiber et al. 2012). In temperate climate forages up to 60 mg phenols/g DM can be found, such as e.g. in *Lotus pedunculatus* (big trefoil; 61.0 mg/g DM), but most plants are not exceeding some 30 mg phenols/g DM phenols, like e.g. *Hedysarum coronarium* (sulla; 33.0 mg/g DM) (Terrill et al. 1992). By contrast, some tropical forages have phenol levels of > 200 mg/g DM (Jayanegara

https://doi.org/10.17221/118/2017-CJAS

et al. 2012). Phenol contents of up to 50 mg/g DM may have positive effects on fertility, absorption of essential amino acids and performance in sheep, while dosages > 50 mg/g dietary DM may decrease DM intake and performance (e.g. Aerts et al. 1999). Wischer et al. (2013) found a CH_4 mitigating effect when testing pure catechin at a dosage of 0.7 mg/g basal diet DM but could not confirm this result in a second study. In the present study, dosages of 0.5, 5.0, and 50 mg/g basal diet DM were chosen in order to determine the range of the lowest concentration that is still effective in CH_4 reduction.

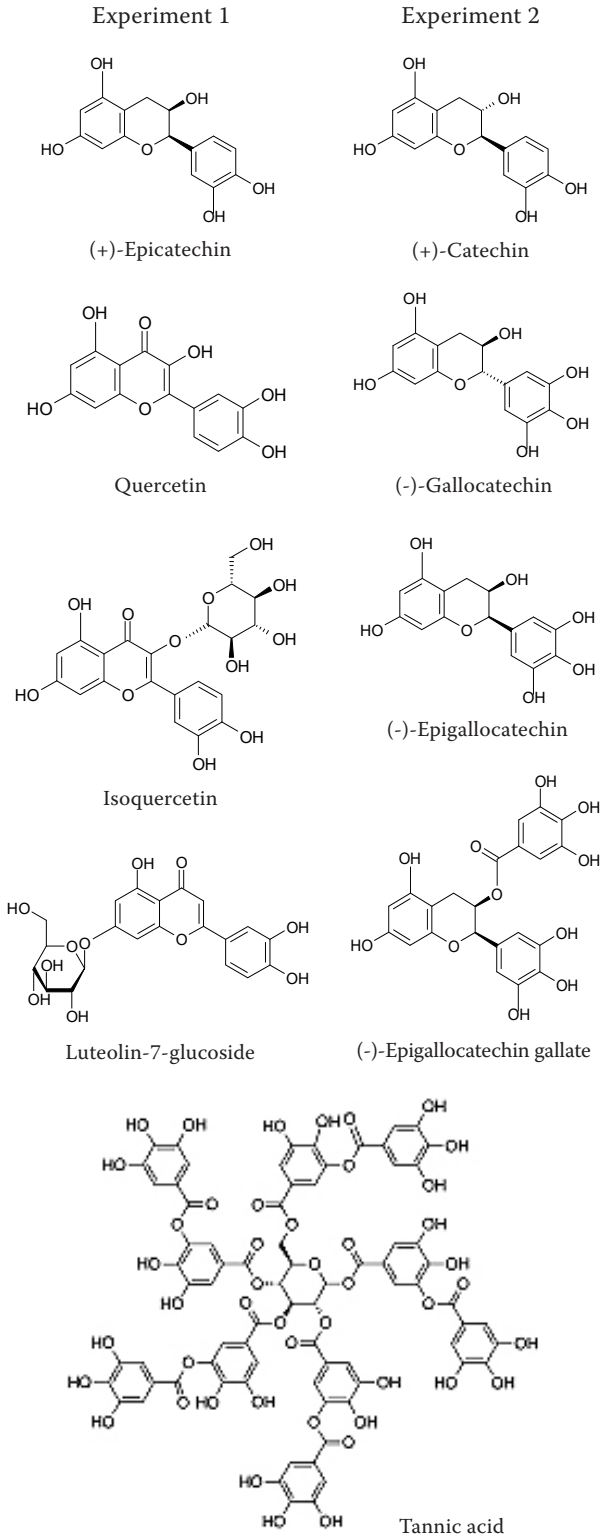

Figure 1. Chemical structure of the compounds investigated in the two experiments

The basal diet consisted of ryegrass (*Lolium multiflorum*) hay prepared from the first cut obtained from a Swiss plant breeder. Within each experiment the same batch of ryegrass hay was used, both in the diet of the donor cow and as basal diet for all *in vitro* incubations. With this procedure, always the hay the microbes were already adapted to was used for the *in vitro* approach. According to proximate and total phenol analysis made in triplicate (AOAC 1997; Jayanegara et al. 2011), the batch of hay used in Experiment 1 contained (per g DM): organic matter 910 mg, crude protein 166 mg, ether extract 38 mg, neutral detergent fibre 526 mg, total extractable phenols 8.2 mg. In the batch of hay used in Experiment 2, the corresponding values were 939, 51, 15, 618, and 1.5 mg.

In both experiments, in each of four different runs the negative control (basal diet alone) was included in two syringes (replicates), while the syringes containing the three levels of each of the four test compounds and the positive control (tannic acid) were included once per each run. This resulted in a total of 68 (4 × 17) incubations per experiment.

In vitro incubations. The Hohenheim Gas Test method was used for the *in vitro* incubations. The system is operated with rumen fluid and buffer as described by Menke and Steingass (1988). Different from that, modified syringes with two outlets were employed (Soliva and Hess 2007) which allowed the direct collection of fermentation gas samples. The rumen fluid used for the individual runs of the two experiments was collected before the morning feeding on eight different days from a rumen-cannulated lactating Brown Swiss cow (approval number ZH 38/14 of the Zürich Cantonal Veterinary Office). The cow was multiparous, had an average live weight of 625 kg, an average milk yield of 7000 kg/year and was in the last and first third of its lactation when rumen fluid was collected for Experiments 1 and 2, respectively. On average across all collections, the rumen fluid had a pH of 6.9 ± 0.1 and it contained per ml 12.9 ± 6.9 × 10^9 bacteria and 113 ± 58 × 10^3 protozoa whereof 98.4%

https://doi.org/10.17221/118/2017-CJAS

were Entodiniomorphs. Accordingly, the rumen fluid used in the incubations was in a range typical for a normal rumen function.

During the time of Experiment 1, the cow received ryegrass hay at *ad libitum* access and daily 0.5 kg of dairy concentrate (UFA 149; UFA AG, Switzerland). During Experiment 2, the diet consisted of ryegrass hay and silage (1 : 1) provided at *ad libitum* access and daily 4 kg of dairy concentrate (UFA Prima F 142; UFA AG). The proximate composition per kg of diet was: organic matter 904 and 879 g, crude protein 188 and 103 g, ether extract 39 and 30 g, neutral detergent fibre 476 and 694 g for Experiments 1 and 2, respectively. The differences found between experiments in the negative control data are likely due the different batches of hay used as basal diet. Additionally, variation in the diet of the donor cow might have changed the properties of the rumen fluid. The cow had free access to water.

The syringes were prepared by filling them with 200 mg DM of the basal diet and the respective amount of the pulverised test compounds. The rumen fluid was strained through four layers of gauze. Afterwards, one part of rumen fluid was combined with two parts of preheated Menke buffer (Menke and Steingass 1988). This formed the incubation fluid, whereof 30 ml each were put into the syringes. The syringes were put in a rotor and incubated for 24 h in a drying cabinet at 39°C. Directly afterwards, the gas volume was determined by readings made on the calibrated scale printed onto the syringes. Then the incubation fluid was removed through one outlet. In these samples, pH (to check sufficient buffering) and NH_3 concentrations were determined with a potentiometer (Model 632 and Model 713; Metrohm, Switzerland) equipped with different electrodes. Due to a failure of the device, it was not possible to measure NH_3 concentrations in Experiment 2. Short-chain fatty acids (SCFA) were analysed in the incubation fluid by high-performance liquid chromatography (HPLC) (LaChrom, L-7000 series, Hitachi Ltd., Japan (Experiment 1) and Agilent 6890N, Agilent Technologies, USA (Experiment 2)) following Ehrlich et al. 1981. Bürker counting chambers (Blau Brand, Germany) were used for microbe counting. These had a depth of 0.02 mm for bacteria and 0.1 mm for protozoa. Hayem solution ($HgCl_2$ 9 mmol/l, Na_2SO_4 176 mmol/l, NaCl 86 mmol/l) and diluted formaldehyde (0.04 mmol/1 w/v in water) were used for fixing bacteria and protozoa, respectively, to facilitate counting. Holotrich and entodiniomorphic protozoa were distinguished. From the remaining fermentation gas, samples were obtained using a sampling injector syringe through an airtight septum covering the second outlet of the pistons (Soliva and Hess 2007). The concentration of CH_4 was analysed by a gas chromatograph (model 5890, series II, Hawlett Packard, USA) equipped with a flame ionisation detector (used for CH_4) and a thermal conductivity detector (used for H_2, CO_2). The column used was a Carboxen-1000, 4.5 m × 3.2 mm (2.1 mm inner diameter), and the carrier gas was argon. *In vitro* organic matter digestibility (IVOMD, %) was calculated according to Menke and Steingass (1988) as 148.8 + 8.893 × gas production (ml) + 0.448 × crude protein (g/kg DM) + 0.651 × total ash (g/kg DM).

Statistical analysis. Data from both experiments (n = 4 per treatment; n = 3 for catechin in Experiment 2) were analysed separately with the Mixed procedure of the SAS software (Statistical Analysis System, Version 9.4). Model 1 considered test compound as fixed effect and incubation run as random effect. For that, the non-supplemented negative control was compared with the respective four different test compounds and tannic acid. This was done separately per dosage of supplementation (either 0.5, 5 or 50 mg/g). For Model 2, dosage was considered as fixed effect and incubation run as random effect. In Model 2, the non-supplemented negative control was considered also as a dosage thus yielding four levels (0, 0.5, 5, and 50 mg/g). Analyses with Model 2 were done separately for each test compound. Multiple comparisons among means were carried out with the Tukey-Kramer method. Normal distribution was examined graphically by plotting the distribution of residuals. Tables display Least Squares Means, standard errors of the mean (SEM) for each level of supplementation, and P-values for the effects of either phenolic compound within dosage or dosage within phenolic compound. Chemical structures were drawn with MDL ISIS^{TM}/Draw (Version 2.5 SP4). Values deviating from the average by more than 2 standard deviations were excluded.

RESULTS

In Experiment 1, the amount of fermentation gas produced and IVOMD were not influenced by the test compounds at the two lower dosages. There was a trend for both variables (P = 0.08) for an effect with 50 mg/g DM when compared to the non-supplemented negative control (Table 1). However, there was an increase (P < 0.05) in gas formation and IVOMD with the highest dosages

https://doi.org/10.17221/118/2017-CJAS

Table 1. Effect of phenolic compounds and their dosage on the production of fermentation gases and on microbial counts during 24 h of fermentation *in vitro*[1] (Experiment 1)

Variable	Dosage (mg/g)	Polyphenol treatment					SEM[4]	*P*-value (compound)
		tannic acid[2]	epicatechin	quercetin	isoquercetin	luteolin-7--glucoside		
Fermentation gas volume (ml)	0[3]	52.8^{y}						
	0.5	54.0	51.9	56.1	55.6^{xy}	56.9^{xy}	1.78	0.220
	5	52.0	54.1	54.9	55.8^{xy}	56.2^{xy}	1.62	0.350
	50	49.4	56.6	56.5	59.5^{x}	57.4^{x}	2.46	0.077
P-value (dosage)		0.689	0.258	0.160	0.018	0.028		
In vitro organic matter digestibility (%)	0[3]	75.1^{y}						
	0.5	76.2	74.3	78.1	77.6^{xy}	78.8^{xy}	1.58	0.220
	5	74.4	76.3	77.0	77.8^{xy}	78.2^{xy}	1.44	0.350
	50	72.1	78.5	78.4	81.9^{x}	79.2^{x}	2.18	0.077
P-value (dosage)		0.689	0.258	0.160	0.018	0.028		
CH_4 volume (ml)	0[3]	8.15^{a,x}						
	0.5	7.80^{x}	7.69	8.08	8.50	8.11^{x}	0.368	0.251
	5	7.43^{xy}	7.55	7.62	7.98	7.90^{xy}	0.279	0.086
	50	6.29^{b,y}	7.62^{a}	7.37^{ab}	7.91^{a}	7.29^{ab,y}	0.359	0.001
P-value (dosage)		0.004	0.184	0.031	0.353	0.018		
CH_4/total gas (ml/ ml)	0[3]	15.5^{a,x}						
	0.5	14.4^{b,y}	14.8^{ab,x}	14.4^{b,y}	15.3^{ab}	14.3^{b,y}	0.51	0.006
	5	14.2^{b,y}	14.0^{b,y}	13.9^{b,yz}	14.3^{b}	14.1^{b,y}	0.45	< 0.001
	50	12.7^{b,z}	13.5^{b,y}	13.1^{b,z}	13.3^{b}	12.7^{b,z}	0.47	< 0.001
P-value (dosage)		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		
NH_3 (μmol/ml incubation fluid)	0[3]	13.0^{a,x}						
	0.5	12.6^{x}	13.4	12.3	12.3	12.4^{xy}	0.69	0.131
	5	12.8^{x}	12.9	12.6	12.2	12.4^{xy}	0.64	0.552
	50	11.0^{b,y}	12.6^{ab}	12.4^{ab}	12.3^{ab}	11.5^{b,y}	0.71	0.006
P-value (dosage)		0.004	0.486	0.324	0.132	0.026		
Bacteria (10^9/ml incubation fluid)	0[3]	2.52^{ab,y}						
	0.5	2.40	2.87	2.54	2.35	2.54^{xy}	0.213	0.580
	5	2.54^{ab}	3.06^{b}	2.06^{ab}	2.37^{ab}	3.35^{a,x}	0.281	0.044
	50	2.62	2.87	2.27	2.35	2.58^{xy}	0.314	0.805
P-value (dosage)		0.956	0.585	0.387	0.874	0.040		
Total protozoa (10^3/ml incubation fluid)	0[3]	21.7^{x}						
	0.5	23.9	17.2	18.6	23.9	11.9^{y}	2.84	0.024
	5	22.9	16.7	22.9	20.5	24.8^{x}	2.96	0.091
	50	16.7	22.0	21.5	15.3	20.1^{xy}	2.89	0.383
P-value (dosage)		0.061	0.363	0.569	0.212	0.006		
Entodiniomorph protozoa (% of total)	0[3]	88.8^{ab}						
	0.5	94.2	89.7^{xy}	82.6	95.0	96.9	4.14	0.115
	5	87.6	96.9^{x}	88.1	90.0	89.5	4.71	0.686
	50	97.2	82.4^{y}	93.4	88.1	97.2	3.88	0.083
P-value (dosage)		0.285	0.051	0.465	0.718	0.101		

[1]values are averages of four replicates obtained from independent incubations except of negative control (n = 8)

[2]tannic acid acted as positive control

[3]0-dosage (negative control without supplementation) that was always included when comparing different means

[4]standard error of the mean

[a–c]means within row with different superscript differ (P < 0.05) for compound effect

[x–z]means within column with different superscript differ (P < 0.05) for dosage effect

https://doi.org/10.17221/118/2017-CJAS

Table 2. Effect of phenolic compounds and their dosage on the production of short-chain fatty acids (SCFA) during 24 h of fermentation *in vitro*[1] (Experiment 1)

Variable	Dosage (mg/g)	Polyphenol treatment					SEM[4]	*P*-value (compound)
		tannic acid[2]	epicatechin	quercetin	isoquercetin	luteolin-7--glucoside		
SCFA (mmol)	0[3]	69.4						
	0.5	69.2	69.2	74.0	71.4	74.4	2.25	0.113
	5	70.2	72.3	73.5	73.0	74.6	2.31	0.289
	50	69.8	74.8	74.3	74.5	75.4	2.13	0.046
P-value (dosage)		0.982	0.130	0.068	0.178	0.053		
Acetate (mmol/mol SCFA)	0[3]	50.0^{y}						
	0.5	50.7	49.6	53.7	52.0^{xy}	53.7^{xy}	1.95	0.109
	5	50.8	52.1	53.3	53.8^{xy}	54.5^{xy}	2.00	0.165
	50	51.0	53.6	54.4	55.4^{x}	55.2^{x}	1.94	0.034
P-value (dosage)		0.921	0.183	0.070	0.046	0.020		
Propionate (mmol/mol SCFA)	0[3]	12.9^{x}						
	0.5	12.7	12.9^{x}	13.8	12.9	13.7	0.64	0.413
	5	13.1	13.5^{xy}	13.6	12.6	13.2	0.65	0.732
	50	12.9	14.8^{y}	13.5	12.7	13.4	0.57	0.091
P-value (dosage)		0.890	0.024	0.403	0.816	0.636		
n-Butyrate (mmol/mol SCFA)	0[3]	4.30						
	0.5	4.21	4.45	4.58	4.62	4.88	0.259	0.282
	5	4.58	4.51	4.56	4.58	4.75	0.272	0.792
	50	4.43	4.30	4.43	4.68	4.84	0.281	0.590
P-value (dosage)		0.592	0.675	0.749	0.505	0.173		
iso-Butyrate (mmol/mol SCFA)	0[3]	0.913						
	0.5	0.528	0.930	0.604	0.495	0.719	0.1626	0.097
	5	0.626	0.947	0.680	0.686	0.794	0.1640	0.473
	50	0.420	0.821	0.524	0.523	0.669	0.1593	0.102
P-value (dosage)		0.059	0.857	0.156	0.056	0.385		
n-Valerate (mmol/mol SCFA)	0[3]	0.602						
	0.5	0.499	0.522	0.488	0.665	0.600	0.0838	0.133
	5	0.548	0.521	0.554	0.605	0.501	0.0706	0.806
	50	0.449	0.603	0.568	0.623	0.629	0.0777	0.228
P-value (dosage)		0.183	0.612	0.273	0.842	0.515		
iso-Valerate (mmol/mol SCFA)	0[3]	0.650^{y}						
	0.5	0.615	0.759	0.835^{x}	0.808	0.795	0.1092	0.147
	5	0.561	0.747	0.873^{x}	0.769	0.846	0.1232	0.175
	50	0.563	0.776	0.843^{x}	0.597	0.675	0.1021	0.089
P-value (dosage)		0.810	0.270	0.003	0.292	0.155		

[1]values are averages of four replicates obtained from independent incubations except of negative control (n = 8)
[2]tannic acid acted as positive control
[3]0-dosage (negative control without supplementation) that was always included when comparing different means
[4]standard error of the mean
$^{x–z}$means within column with different superscript differ (P < 0.05) for dosage effect

of isoquercetin and luteolin-7-glucoside when compared to the zero dosage (non-supplemented negative control). The absolute amount of CH_4 (ml) was reduced (P < 0.05) by the highest dosage of tannic acid. Additionally to tannic acid, there was a dosage effect with luteolin-7-glucoside with a decrease in CH_4 amounts with increasing dosage. Relative to the negative control, tannic acid as well as epicatechin, quercetin, isoquercetin, and luteolin-7-glucoside reduced (P < 0.05) the

https://doi.org/10.17221/118/2017-CJAS

amount of CH_4 produced in relation to total gas produced with dosages of 0.5 mg/g (only tannic acid, quercetin, and luteolin-7-glucoside), 5 mg/g, and 50 mg/g DM, and this in a dosage depending way ($P < 0.05$). In detail, compared with the negative control the maximal declines amounted to 17.9, 17.7, 15.5, 14.1, and 12.8% with the 50 mg/g DM dosage of luteolin-7-glucoside, tannic acid, quercetin, isoquercetin, and epicatechin, respectively. The incubation fluid NH_3 concentration decreased ($P < 0.05$) with the 50 mg dosage of luteolin-7-glucoside (–11.5%) and tannic acid (–15.3%) compared to the negative control. The supplementation with different phenolic compounds had no effect on variables describing microbial counts. However, there was a dosage effect ($P < 0.05$) with luteolin-7-glucoside, with reduced numbers of total bacteria (5 mg/g DM dosage) and total protozoa (0.5 mg/g DM dosage) (Table 1). The amount of SCFA produced and the SCFA profile were mostly not influenced by the type and dosage of phenolic compounds (Table 2). However, there were general treatment effects ($P < 0.05$) with the highest dosage in SCFA amount and acetate proportion of total SCFA concentration, and a trend ($P = 0.10$) in proportions of propionate, *iso*-butyrate, and *iso*-valerate. In these cases, no differences ($P > 0.10$) between individual treatments were identified by the multiple comparisons among means. There were also occasional dosage effects ($P < 0.05$): increase of acetate proportion with isoquercetin and luteolin-7-glucoside, of propionate proportion with epicatechin, and of *iso*-valerate proportion with quercetin (Table 2).

In Experiment 2, gallocatechin and epigallocatechin gallate (provided at 5 and 50 mg/g DM) as well as tannic acid (50 mg/g DM) lowered ($P < 0.05$) fermentation gas formation and IVOMD compared to the negative control, and this in a dosage dependent way (the latter also with epigallocatechin) (Table 3). Compared to the negative control, the absolute amount of CH_4 was reduced ($P < 0.05$) by tannic acid and epigallocatechin gallate when provided at 50 mg/g DM, and there were dosage effects ($P < 0.05$) with these two compounds and with epigallocatechin. When CH_4 was related to total gas, only tannic acid was effective in reduction ($P < 0.05$) at the highest dosage, and dosage effects were found with the same compounds as those with absolute CH_4 amount. The supplementation with different phenolic compounds had no effect ($P > 0.10$) on microbial counts (Table 3). When supplementing the compounds at 50 mg/g DM, epigallocatechin lowered ($P < 0.05$) total SCFA concentration, as well as propionate and *n*-butyrate proportions of total SCFA compared to the negative control (Table 4). Furthermore, 50 mg of tannic acid and of any flavonoid (for epigallocatechin and epigallocatechin gallate also the 5 mg dosage) reduced ($P < 0.05$) proportions of *n*-valerate and *iso*-valerate (except catechin). Various compounds exhibited dosage effects ($P < 0.05$) in either total SCFA concentration or molar proportions of individual SCFA, with always the highest dosage being most effective.

DISCUSSION

Natural occurrence and properties of the compounds tested. The eight flavonoids selected for the present study to test their effect on ruminal digestion are prevalent in nature and occur in a number of plants, whereof certain plants are particularly rich in these compounds. Quercetin (Experiment 1) is a yellow natural colorant with anti-oxidative and anti-inflammatory properties (Hatahet et al. 2016). One plant rich in quercetin is buckwheat (Fabjan et al. 2003) which also contains substantial amounts of the flavonole rutin, which was investigated earlier (Leiber et al. 2012). Isoquercetin (Experiment 1) occurs for instance in apples and onions (Erlund 2004). Luteolin-7-glucoside (Experiment 1) can be found in spices like peppermint, oregano, and celery seed (Bhagwat et al. 2014). Epicatechin (Experiment 1), catechin, gallocatechin, epigallocatechin, and epigallocatechin gallate (Experiment 2) are elements of a number of CT but also occur in their free form for example in grape or tea (Manach et al. 2004). The latter five compounds in free form are known to have strong antioxidant properties (Scola et al. 2010). Finally, the compound used as positive control, tannic acid, is no flavonoid and is used as tannic substance in the leather industry. It is the by far largest compound tested in the present study (Figure 1) and can be released as a sub-unit from HT during ruminal degradation (Nelson et al. 1995), whereas CT are resistant against digestion in the rumen (Makkar et al. 1995). Therefore, the five flavonoids forming sub-units of the CT would not act as free compounds in the rumen when feeds containing CT are consumed and their direct effects

https://doi.org/10.17221/118/2017-CJAS

Table 3. Effect of phenolic compounds and their dosage on the production of fermentation gases and on microbial counts during 24 h of fermentation *in vitro*[1] (Experiment 2)

Variable	Dosage (mg/g)	Polyphenol treatment					SEM[4]	*P*-value (compound)
		tannic acid[2]	catechin	gallocatechin	epigallo-catechin	epigallo-catechin gallate		
Fermentation gas volume (ml)	0[3]	47.6^{a,x}						
	0.5	47.9^{x}	46.1	46.6^{xy}	45.0^{xy}	45.0^{xy}	1.57	0.066
	5	45.1^{ab,xy}	45.8^{ab}	44.0^{b,xy}	44.6^{ab,y}	43.8^{b,y}	1.94	0.007
	50	42.1^{c,y}	46.9^{ab}	42.5^{c,y}	46.0^{ab,xy}	43.3^{bc,y}	2.35	< 0.001
P-value dosage		< 0.001	0.437	0.009	0.027	0.008		
In vitro organic matter digestibility (%)	0[3]	63.4^{a,x}						
	0.5	63.7^{x}	62.2	62.6^{xy}	61.2^{xy}	61.2^{xy}	1.40	0.066
	5	61.3^{ab,xy}	61.8^{ab}	60.3^{b,xy}	60.8^{ab,y}	60.0^{b,y}	1.72	0.007
	50	58.6^{c,y}	62.9^{ab}	58.9^{c,y}	62.0^{ab,xy}	59.6^{bc,y}	2.20	< 0.001
P-value (dosage)		< 0.001	0.437	0.009	0.027	0.008		
CH_4 volume (ml)	0[3]	5.81^{a,x}						
	0.5	5.94^{x}	5.88	6.02	5.91^{x}	5.97^{x}	0.25	0.825
	5	5.50^{x}	5.75	5.68	5.74^{x}	5.73^{x}	0.24	0.715
	50	4.63^{c,y}	5.41^{ab}	5.31^{ab}	5.37^{ab,y}	5.08^{bc,y}	0.33	< 0.001
P-value (dosage)		< 0.001	0.315	0.074	0.002	0.002		
CH_4/total gas (ml/ml)	0[3]	12.3^{a,x}						
	0.5	12.4^{x}	12.7	12.9	13.1^{x}	13.2^{x}	0.38	0.062
	5	12.2^{x}	12.6	12.9	12.9^{xy}	13.1^{xy}	0.40	0.190
	50	11.0^{b,y}	11.5^{ab}	12.5^{a}	11.7^{ab,z}	11.7^{ab,z}	0.41	0.005
P-value (dosage)		0.002	0.186	0.191	0.002	0.004		
Bacteria (10^9/ml incubation fluid)	0[3]	2.46						
	0.5	2.29	2.13	2.25	1.90	1.85	0.196	0.122
	5	2.29	2.04	1.92	2.21	2.17	0.258	0.597
	50	2.46	2.51	2.42	1.87	2.13	0.231	0.179
P-value (dosage)		0.843	0.373	0.260	0.078	0.157		
Total protozoa (10^3/ml incubation fluid)	0[3]	44.6						
	0.5	56.8	47.4	37.0	40.6	42.5	8.76	0.165
	5	47.4	41.3	40.0	45.8	28.4	7.06	0.167
	50	43.1	45.6	49.2	40.3	38.2	6.76	0.487
P-value (dosage)		0.254	0.866	0.207	0.777	0.061		
Entodiniomorph protozoa (% of total)	0[3]	95.9						
	0.5	97.2	96.1	97.1	96.5	96.0	1.76	0.985
	5	96.8	94.6	95.9	91.4	94.9	1.50	0.162
	50	95.4	95.4	92.6	92.4	94.7	2.54	0.641
P-value (dosage)		0.854	0.889	0.425	0.084	0.942		

[1]values are averages of four replicates obtained from independent incubations except of negative control (n = 8)
[2]tannic acid acted as positive control
[3]0-dosage (negative control without supplementation) that was always included when comparing different means
[4]standard error of the mean
[a–c]means within row with different superscript differ (P < 0.05) for compound effect
[x–z]means within column with different superscript differ (P < 0.05) for dosage effect

on ruminal fermentation, methane and ammonia formation were thus tested for the first time (except catechin) (Becker et al. 2014) in the present study.

Compound effects on ruminal nutrient fermentation. The primary aim of the present study was to identify flavonoids with methane and NH_3

https://doi.org/10.17221/118/2017-CJAS

Table 4. Effect of phenolic compounds and their dosage on the production of short-chain fatty acids (SCFA) during 24 h of fermentation *in vitro*[1] (Experiment 2)

Variable	Dosage (mg/g)	Polyphenol treatment					SEM[4]	*P*-value (compound)
		tannic acid[2]	catechin	gallocatechin	epigallo-catechin	epigallo-catechin gallate		
SCFA (mmol)	0[3]	70.3^{a,x}						
	0.5	70.9^{x}	71.0	68.9	69.3^{x}	69.9	2.07	0.100
	5	70.8^{x}	68.7	68.9	69.2^{x}	70.8	1.98	0.079
	50	67.9^{ab,y}	68.6^{ab}	71.3^{a}	61.5^{b,y}	67.2^{ab}	3.42	0.042
P-value (dosage)		0.006	0.024	0.116	0.012	0.322		
Acetate (mmol/mol SCFA)	0[3]	47.5						
	0.5	48.2	48.4	46.9	47.3	47.8	1.41	0.083
	5	48.0	47.0	47.0	47.7	48.3	1.38	0.166
	50	47.1	47.4	49.0	42.5	46.3	2.48	0.124
P-value (dosage)		0.270	0.154	0.052	0.050	0.621		
Propionate (mmol/mol SCFA)	0[3]	13.5^{a,x}						
	0.5	13.7^{x}	13.6	13.3	13.3^{x}	13.4^{xy}	0.30	0.356
	5	13.7^{x}	13.2	13.3	13.2^{x}	13.4^{xy}	0.33	0.219
	50	12.6^{ab,y}	13.0^{a}	13.9^{a}	11.8^{b,y}	12.6^{ab,y}	0.47	0.005
P-value (dosage)		< 0.001	0.093	0.154	0.002	0.029		
n-Butyrate (mmol/mol SCFA)	0[3]	7.44^{a,x}						
	0.5	7.40^{x}	7.38^{xy}	7.14^{xy}	7.17^{x}	7.20	0.653	0.101
	5	7.46^{x}	7.05^{xz}	7.07^{yx}	7.15^{x}	7.50	0.607	0.017
	50	6.76^{ab,y}	6.72^{ab,z}	7.02^{ab,y}	6.39^{b,y}	6.95^{ab}	0.675	0.006
P-value (dosage)		< 0.001	< 0.001	0.025	0.001	0.193		
iso-Butyrate (mmol/mol SCFA)	0[3]	0.355						
	0.5	0.320	0.294	0.299	0.259	0.307	0.0664	0.809
	5	0.310	0.256	0.274	0.251	0.355	0.0648	0.536
	50	0.267	0.294	0.335	0.210	0.355	0.0745	0.427
P-value (dosage)		0.065	0.300	0.644	0.149	0.922		
n-Valerate (mmol/mol SCFA)	0[3]	0.540^{a,x}						
	0.5	0.503^{x}	0.478^{xy}	0.504^{x}	0.410^{x}	0.463^{xy}	0.0478	0.268
	5	0.516^{x}	0.453^{y}	0.472^{xy}	0.402^{x}	0.499^{xy}	0.0425	0.190
	50	0.420^{b,y}	0.374^{bc,y}	0.382^{b,y}	0.232^{c,y}	0.372^{b,y}	0.0391	< 0.001
P-value (dosage)		< 0.001	0.002	0.003	< 0.001	0.023		
iso-Valerate (mmol/mol SCFA)	0[3]	0.934^{a,x}						
	0.5	0.838^{xy}	0.860^{xy}	0.840^{x}	0.802^{x}	0.758^{xy}	0.0994	0.425
	5	0.811^{ab,y}	0.788^{ab,y}	0.817^{ab,x}	0.466^{c,y}	0.679^{bc,xy}	0.0869	< 0.001
	50	0.656^{bc,z}	0.768^{ab,y}	0.624^{bc,y}	0.419^{c,y}	0.545^{bc,y}	0.1105	< 0.001
P-value (dosage)		< 0.001	0.009	< 0.001	< 0.001	0.007		

[1]values are averages of four replicates obtained from independent incubations except of negative control (n = 8)
[2]tannic acid acted as positive control
[3]0-dosage (negative control without supplementation) that was always included when comparing different means
[4]standard error of the mean
$^{a–c}$means within row with different superscript differ ($P < 0.05$) for compound effect
$^{x–z}$means within column with different superscript differ ($P < 0.05$) for dosage effect

mitigating properties. In order to have potential to be considered for sustainable ruminant nutrition, diets should not exert substantial adverse side-effects on ruminal fermentation and thus energy supply to the host. As indicators for the intensity of fermentation, production of fermentation gas

https://doi.org/10.17221/118/2017-CJAS

and SCFA were measured and IVOMD was calculated. Further information about ruminal nutrient formation was obtained by determining SCFA profile and microbial counts per unit of fermentation fluid. In Experiment 1, two out of the compounds tested, luteolin-7-glucoside and isoquercetin, tended even to increase fermentation gas volume and IVOMD when added to ryegrass, while epicatechin and quercetin did not influence these parameters. Berger et al. (2015) found that quercetin aglycol and rutin (both as sources of quercetin) provided at 50 and 100 µmol of quercetin equivalents per litre (corresponding to 25.4 and 50.7 mg/g DM) did not influence gas production in Hohenheim Gas Test, either. In a subsequent *in vivo* study with 10 and 50 mg of quercetin equivalents per kg of body weight (corresponding to 1.8 and 9.1 mg/g DM) administrated via rumen fistula, quercetin did not influence SCFA concentration or profile. An ethanol extract from portulaca rich in flavonoids, containing luteolin and quercetin, was found to enhance *in vitro* gas and SCFA production (Wang et al. 2013). Different from these flavonoids, in Experiment 2, tannic acid, gallocatechin, and epigallocatechin gallate lowered fermentation gas formation and IVOMD while epigallocatechin lowered SCFA production. The decrease in gas and SCFA formation with tannic acid was not significant in Experiment 1. Although the same basal diet type was used, the differences in the response to the negative control and tannic acid (positive control) in Experiment 1 and 2 could be due to differences in the rumen fluid due to different diets fed to the donor cow but also due to the different chemical composition of the basal diet (ryegrass hay) used for the incubations. There is some evidence that gross nutrients like crude protein and fibre can interact with bioactive compounds and make them physically differently available for the microbes (cf. Cieslak et al. 2014). The influence of the three CT-related flavanols (gallocatechin, epigallocatchin, and epigallocatchin gallate) on fermentation is consistent with anti-nutrititional effects found with higher levels of CT, as CT can bind to feed proteins but also enzymes and carbohydrates thus reducing their ruminal degradation (reviewed by McSweeney et al. 2001). Also HT, and thus tannic acid, have this property (McSweeney et al. 2001). Besides the influence on SCFA concentration, also the SCFA profile was affected especially by tannic acid, catechin, gallocatechin, epigallocatechin, epigallocatechin gallate, and quercetin which all influenced *iso*-valerate. As short-chain *iso*-acids like *iso*-valerate are microbial breakdown products of branched-chain amino acids (Gorosito et al. 1985), a lower proportion of these SCFA coincides with the lower ammonia concentration found.

Compound effects on ruminal methane and ammonia formation. The effects of the compounds on CH_4 emission from the fermentation of the ryegrass hay have to be distinguished into those affecting the absolute amount (tannic acid and epigallocatechin gallate) and those reducing CH_4 in relation to total fermentation gas (tannic acid, epicatechin, quercetin, isoquercetin, luteolin-7-glucoside). The latter variable is an indicator for the amount of CH_4 emitted per unit of net energy available to the animal and, in a wider sense, even per unit of milk or meat produced as performance depends on net energy supply.

Overall, in CH_4 mitigation out of the eight flavonoids tested, luteolin-7-glucoside was superior to the other flavonoids and similarly effective as tannic acid. This result is underlined by the reduction of absolute CH_4 amount found with a luteolin containing extract of portulaca (Wang et al. 2013). In the present experiment, the reduction in CH_4 was not accompanied by a shift in acetate/propionate ratio or a reduction in protozoal counts like in previous studies reviewed by Patra et al. (2011). However, effects of particular secondary compounds can differ depending on their chemical structure and dosage (Cieslak et al. 2014). Similarly, phenols from *Sanguisorba officinalis* were found to affect *in vitro* ruminal CH_4 production whereas the bioactive components had no negative impact on *in vitro* rumen microbial populations, dry matter digestibility, and SCFA concentration (Cieslak et al. 2016). This indicates that luteolin-7-glucoside and tannic acid used in the present study might have selectively impaired the methanogenic archaeal population (Patra et al. 2011), which was not quantified separately. Some other test compounds were similarly effective as the flavon luteolin-7-glucoside in mitigating the CH_4-to-total gas ratio. In the cases of epicatechin and isoquercetin this happened by enhancing fermentation without concomitant increase in methane formation. From the five CT-related flavanols, only catechin and gallocatechin had no effect on the CH_4/total gas ratio. For catechin, this was in contrast to the *in vitro* findings of Becker et al. (2014), but in their study much higher dosages (between 36 and 4.6 mg/20 ml of rumen fluid) and a different substrate (polylactate) were used. The present results indicate that at least epicatechin, among the five sub-units of CT investigated in the present study, helps explaining the known

https://doi.org/10.17221/118/2017-CJAS

methane mitigating effects of CT-containing diets (Jayanegara et al. 2012).

Excessive dietary protein degradation to NH_3 by rumen microorganisms is an inefficient way to generate metabolisable protein for the host and may be at least partially inhibited by the complexation abilities of phenolic compounds (McSweeney et al. 2001). Apart from tannic acid (reduction by up to 15%), only luteolin-7-glucoside reduced NH_3 concentration in the incubation fluid (by up to 12%). This effect was even larger than the 8% reduction found with the same dosages of rutin (Leiber et al. 2012). Also Wang et al. (2013) described lowering of the NH_3 concentration when supplementing luteolin via the portulaca extract. Phenols can reduce the accessibility of dietary protein to the microbes by forming complexes but may also directly inhibit proteolytic bacteria or their enzymatic activity (Patra et al. 2011). This results in a reduced protein degradation and, consequently, a lower NH_3 concentration in rumen fluid. As no data were available from Experiment 2, possible NH_3-mitigating properties of four of the five CT-related flavonoids remain to be determined. Especially the polyphenols occurring in tannins (tannic acid and the flavanols catechin, epicatechin, gallocatchin, epigallocatechin, and epigallocatechin gallate) were expected to be able to diminish NH_3 release from forage as has been shown in a large number of studies reviewed by McSweeney et al. (2001).

Dosage effects. There were dosage effects by many of the test compounds in a number of variables. Most effects on ruminal CH_4 emission and NH_3 concentration were found with the highest dosage, 50 mg/g DM, some with 5 mg/g DM. In fermentation intensity (i.e. the level of production of fermentation gas and IVOMD), dosage effects were particularly large with epigallocatechin gallate but were also found with gallocatchin, epigallocatechin, isoquercetin, luteolin-7-glucoside, and tannic acid. Dosage effects on the level of reduction of CH_4 emission (measured in respiration chamber) and ruminal NH_3 concentration were also reported by Yang et al. (2017), who was offering diets with 0, 6.5, 13.0 or 26.0 g tannic acid/kg DM to beef cattle.

CONCLUSION

Out of the eight flavonoid compounds tested, luteolin-7-glucoside was most promising. Its supplementation mitigated CH_4 and NH_3 formation in rumen fluid *in vitro* at unchanged fermentation activity. Different from tannic acid, luteolin-7-glucoside mediated its effect against CH_4 formation without compromising fermentation efficiency. However, further *in vitro* and *in vivo* studies with extracts of such plants are needed to find out the most effective sources and dosages.

REFERENCES

Aerts R.J., Barry T.N., McNabb W.C. (1999): Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. Agriculture, Ecosystems and Environment, 75, 1–12.

AOAC (1997): Official Methods of Analysis. 16th Ed. Association of Official Analytical Chemists, Arlington, USA.

Beauchemin K.A., Kreuzer M., O'Mara F., McAllister T.A. (2008): Nutritional management for enteric methane abatement: a review. Australian Journal of Experimental Agriculture, 48, 21–27.

Becker P.M., van Wikselaar P.G., Franssen M.C.R., de Vos R.C.H., Hall R.D., Beekwilder J. (2014): Evidence for a hydrogen-sink mechanism of (+)catechin-mediated emission reduction of the ruminant greenhouse gas methane. Metabolomics, 10, 179–189.

Berger L.M., Blank R., Zorn F., Wein S., Metges C.C., Wolffram S. (2015): Ruminal degradation of quercetin and its influence on fermentation in ruminants. Journal of Dairy Science, 98, 5688–5698.

Bhagwat S., Haytowitz D.B., Holden J.M. (2014): USDA Database for the Flavonoid Content of Selected Foods, Release 3.1. US Department of Agriculture, Agricultural Research Service, Beltsville, USA. Available from https://www.ars.usda.gov/ARSUserFiles/80400525/Data/Flav/Flav_R03-1.pdf (accessed July 17, 2018).

Bjorklund G., Chirumbolo S. (2017): Role of oxidative stress and antioxidants in daily nutrition and human health. Nutrition, 33, 311–321.

Cieslak A., Szumacher-Strabel M., Stochmal A., Oleszek W. (2013): Plant components with specific activities against rumen methanogens. Animal, 7 (Suppl. 2), 253–265.

Cieslak A., Zmora P., Stochmal A., Pecio L., Oleszek W., Pers-Kamczyc E., Szczechowiak J., Nowak A., Szumacher-Strabel M. (2014): Rumen antimethanogenic effect of Saponaria officinalis L. phytochemicals in vitro. The Journal of Agricultural Science, 152, 981–993.

Cieslak A., Zmora P., Matkowski A., Nawrot-Hadzik I., Pers-Kamczyc E., El-Sherbiny M., Bryszak M., Szumacher-Strabel M. (2016): Tannins from Sanguisorba officinalis affect in vitro rumen methane production and fermentation. Journal of Animal and Plant Sciences, 26, 54–62.

Cortes J.E., Moreno B., Pabon M.L., Avila P., Kreuzer M., Hess H.D., Carulla J.E. (2009): Effects of purified con-

https://doi.org/10.17221/118/2017-CJAS

densed tannins extracted from Calliandra, Flemingia and Leucaena on ruminal and postruminal degradation of soybean meal as estimated in vitro. Animal Feed Science and Technology, 151, 194–204.

Crozier A., Clifford M.N., Ashihara H. (2006): Phenols, polyphenols and tannins: an overview. In: Crozier A., Clifford M.N., Ashihara H. (eds): Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet. Blackwell Publishing, 1–24.

Ehrlich G.G., Goerlitz D.F., Bourell J.H., Eisen G.V., Godsy E.M. (1981): Liquid chromatographic procedure for fermentation product analysis in the identification of anaerobic bacteria. Applied and Environmental Microbiology, 42, 878–885.

Erlund I. (2004): Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. Nutrition Research, 24, 851–874.

Fabjan N., Rode J., Kosir I.J., Wang Z., Zhang Z., Kreft I. (2003): Tartary buckwheat (Fagopyrum tataricum Gaertn.) as a source of dietary rutin and quercitrin. Journal of Agricultural and Food Chemistry, 51, 6452–6455.

Field J.A., Lettinga G. (1987): The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. Water Research, 21, 367–374.

Gorosito A.R., Russell J.B., Van Soest P.J. (1985): Effect of carbon-4 and carbon-5 volatile fatty acids on digestion of plant cell wall in vitro. Journal of Dairy Science, 68, 840–847.

Hatahet T., Morille M., Hommoss A., Devoisselle J.M., Muller R.H., Begu S. (2016): Quercetin topical application, from conventional dosage forms to nanodosage forms. European Journal of Pharmaceutics and Biopharmaceutics, 108, 41–53.

Jayanegara A., Wina E., Soliva C.R., Marquardt S., Kreuzer M., Leiber F. (2011): Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis. Animal Feed Science and Technology, 163, 231–243.

Jayanegara A., Leiber F., Kreuzer M. (2012): Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. Journal of Animal Physiology and Animal Nutrition, 96, 365–375.

Leiber F., Kunz C., Kreuzer M. (2012): Influence of different morphological parts of buckwheat (Fagopyrum esculentum) and its major secondary metabolite rutin on rumen fermentation in vitro. Czech Journal of Animal Science, 57, 10–18.

Makkar H.P.S., Becker K., Abel H., Szegletti C. (1995): Degradation of condensed tannins by rumen microbes exposed to quebracho tannins (QT) in rumen simulation technique (RUSITEC) and effects of QT on fermentative processes in the RUSITEC. Journal of the Science of Food and Agriculture, 69, 495–500.

Manach C., Scalbert A., Morand C., Remesy C., Jimenez L. (2004): Polyphenols: food sources and bioavailability. American Society for Clinical Nutrition, 79, 727–747.

McSweeney C.S., Palmer B., McNeill D.M., Krause D.O. (2001): Microbial interactions with tannins: nutritional consequences for ruminants. Animal Feed Science and Technology, 91, 83–93.

Menke K.H., Steingass H. (1988): Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Animal Research and Development, 28, 7–55.

Nelson K.E., Pell A.N., Schofield P., Zinder S. (1995): Isolation and characterization of an anaerobic ruminal bacterium capable of degrading hydrolyzable tannins. Applied and Environmental Microbiology, 61, 3293–3298.

Patra A.K., Min B.R., Saxena J. (2011): Dietary tannins on microbial ecology of the gastrointestinal tract in ruminants. In Patra A.K. (ed.): Dietary Phytochemicals and Microbes. Springer, Dordrecht, the Netherlands, 237–262.

Scola G., Conte D., Spada P.W., Dani C., Vanderlinde R., Funchal C., Salvador M. (2010): Flavan-3-ol compounds from wine wastes with in vitro and in vivo antioxidant activity. Nutrients, 2, 1048–1059.

Soliva C.R., Hess H.D. (2007): Measuring methane emission of ruminants by in vitro and in vivo techniques. In: Makkar H.P.S., Vercoe P.E. (eds): Measuring Methane Production from Ruminants. Springer, Dordrecht, the Netherlands, 15–31.

Terrill T.H., Rowan A.M., Douglas G.B., Barry T.N. (1992): Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. Journal of the Science of Food and Agriculture, 58, 321–329.

Wang D., Huang J., Zhang Z., Tian X., Huang H., Yu Y., Zhang G., Ding J., Huang R. (2013): Influences of Portulaca oleracea extracts on in vitro methane emissions and rumen fermentation of forage. Journal of Food, Agriculture and Environment, 11, 483–488.

Wischer G., Boguhn J., Steingass H., Schollenberger M., Rodehutscord M. (2013): Effects of different tannin-rich extracts and rapeseed tannin monomers on methane formation and microbial protein synthesis in vitro. Animal, 7, 1796–1805.

Yang K., Wei C., Zhao G.Y., Xu Z.W., Lin S.X. (2017): Effects of dietary supplementing tannic acid in the ration of beef cattle on rumen fermentation, methane emission, microbial flora and nutrient digestibility. Journal of Animal Physiology and Animal Nutrition, 101, 302–310.

Received: 2017–11–24
Accepted after corrections: 2018–05–09