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Low concentrations of a polyphenolic extract from pine bark in high–concentrate diets decrease *in vitro* rumen ammonia nitrogen but not methane production

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ABSTRACT

This study was conducted to assess the effects of small supplemental doses of a polyphenolic extract from pine bark (PBE) on CH_4 output and ruminal fermentation parameters when incubated in batch culture with a high–concentrate diet for 24–h. The data from the dietary substrates supplemented with 0.0, 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8% of PBE were evaluated in a randomized complete block design, and compared using ANOVA followed by Tukey's test and polynomial contrasts. Increasing doses of the PBE caused a linear decrease of the NH_3–N concentration ($p < 0.001$), the potentially degradable dry matter (DM) fraction ($p = 0.002$), the partitioning factor ($p = 0.001$), CH_4 production and proportion ($p = 0.001$ and $p = 0.029$, respectively), although only at 6–h, achieving the lowest productions ($p = 0.016$) with 1.5 and 1.8% PBE. In contrast, the PBE linearly increased asymptotic gas production ($p = 0.007$), gas yield ($p = 0.004$), pH ($p = 0.002$) and the short–chain fatty acid concentration ($p < 0.001$) at 24–h. Addition of least 1.5% PBE to high–concentrate diets reduces CH_4 production by 31% at 6–h, whereas NH_3–N concentration is reduced by 31% at 24–h incubations.

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Introduction

Animal farming is challenged by a growing demand for quality meat and milk products and, at the same time, by keeping a steady environmental impact (Malik et al. 2017). The livestock industry contributes about 14.5% to the global greenhouse gases (GHG) emissions, with methane (CH_4) accounting for 44% of these emissions (Gerber et al. 2013) and representing an energy loss of 2–12%. Additionally, up to 50% of nitrogen (N) intake can be lost by degradation to ammonia (NH_3) and excretion via urine (Salem et al. 2015; Brutti et al. 2019), further contributing to GHG emissions because of its potential conversion into nitrous oxide (N_2O; IPCC 2013). It is possible to reduce CH_4 emissions and N excretion (Bhatta et al. 2015) by manipulating the rumen ecosystem through growth promoters, antimicrobials and hormones. However, the negative perception of consumers on chemical additives has intensified research for natural additives such as polyphenols, flavonoids and tannins with the potential of modulating rumen fermentation and rumen–derived products (Balcells et al. 2012; Brutti et al. 2019; Vasta et al. 2019; Purba et al. 2020a).

Flavonoids, such as tannins, are plant polyphenolic secondary metabolites considered as safe for the environment and for the consumer (Jiménez–Peralta et al. 2011; Bhatta et al. 2015), and have the ability to bind proteins and carbohydrates (Deaville et al. 2010). They have also shown antimicrobial properties (Purba et al. 2020a) and have a profound effect on the outcome of ruminal fermentation of ruminant diets (Aderao et al. 2018). Tannins have been considered antinutritional factors as they can reduce DM intake and protein and carbohydrate digestion (Oliveira et al. 2007). However, depending on the source, concentration, and type of tannin applied to ruminant diets, either naturally or supplemented, they can have an anti–methanogenic effect (Malik et al. 2017; Vasta et al. 2019), and improve N use in ruminants by reducing crude protein (CP) degradation (Castro–Montoya et al. 2018), thus improving live weight gain, milk yields and animal fertility and health status (Hatami et al. 2018; Vasta et al. 2019).

Natural polyphenol–rich extracts can be obtained from trees such as acacia (*Acacia mearnsii*), quebracho (*Schinopsis balansae* and *S. lorentzii*) or pine (*Pinus radiata*; García et al. 2016). Chilés forest industry is based on *P. radiata* production, with a cultivated area of 1.6 million hectares, equivalent to one–third of the total *P. radiata* planted globally (Guerrero and Bustamante 2007). Bark represents at least 10% of total pine weight and generates a biomass residue of 1.4–1.5 million tons per year which is generally used to generate electricity by combustion, resulting in negative environmental impacts. However, a polyphenol–rich extract (mixture of flavonoids, stilbenoids and condensed tannins) from *P. radiata* bark (Berg et al. 2009), reduced ammonia nitrogen (NH_3–N) concentration in *in vitro* fermentation by 50%, without affecting diet digestibility or CH_4 production when used at concentrations of 2–4% DM PBE in ruminant forage diets (Vera et al. 2018). Yang et al. (2016) also reported that the supplementation with a moderate concentration (3% DM) of an extract from *P. taeda* bark decreased NH_3–N concentration without affecting CH_4

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production, suggesting that PBE could affect dietary N use efficiency *in vivo*. However, the effects of low concentrations of PBE in cattle concentrate diets containing high proportions of quickly degradable protein has not yet been assessed. The purpose of this batch test was to assess the smallest (< 2% DM) effective dose of a PBE as an additive in high–concentrate ruminant diets to decrease NH_3–N concentration and CH_4 production.

Materials and methods

This experiment was conducted at the Livestock Systems and Nutrition Laboratory of the Universidad de Concepción (UdeC), Chillán, Chile. The care and management of the cows were certified by the animal ethics and welfare committee of the UdeC.

Extract from Pinus radiata bark

The polyphenolic PBE was produced by methanolic extraction at the Technological Development Unit, UdeC, according to Berg et al. (2009). This extract is an aqueous solution (38.0% DM) with a concentration of 133.2 g of total polyphenols/kg DM, or 43.5 g of total tannins (TT)/kg DM. It is mainly composed of flavonoids (luteolin, pinocembrin, catechin, procyanidin, gallocatechin, quercetin and taxifolin) and small amounts of stilbenoids (astringin and piceatannol) and phenolic acid.

Incubation substrates and treatments

The substrates simulated a high–concentrate diet with forages containing high concentrations of quickly degradable protein for cattle. Therefore, the incubation substrates were corn grain, mixed hay (*Lolium perenne* with *Trifolium repens*) and soybean meal in a ratio of 60:20:20, respectively. Treatments were 0 (Control) or 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8% DM basis PBE replacing equivalent amounts of substrate. All ingredients were ground (2 mm; Grain Mill, Breuer, Temuco, Chile) before adding PBE and thereafter mixed. The ingredients and PBE inclusion are listed in Table 1.

Donor animals and batch incubation

Rumen fluid was obtained from two non–lactating rumen–cannulated adult Aberdeen Angus cows (500 kg body weight), fed a diet containing mixed hay (*Lolium perenne* with *Trifolium repens*), ground corn and a vitamin–mineral supplement in a ratio of 70:25:5, respectively, formulated to meet nutritional requirements for maintenance of adult 500–kg cows (NASEM 2016). Animals were fed daily at 7 am and 5 pm Access to fresh water was available at all times.

Two hours after the morning feeding rumen fluid was collected and filtered through four cheesecloth layers and immediately transported to the laboratory in a pre–heated thermal flask (39°C). The inoculum was a blend of rumen fluid and mineral buffer (Menke et al. 1979) in a 1:3 ratio (v/v).

Substrates were weighed (0.5 g) into ANKOM F57 filter bags (Ankom Technology Corp., Macedon NY), and each substrate bag was placed individually in a 50 mL amber glass bottle (Avila et al. 2011). For each dose of PBE (n = 7) and sampling time (6, 12 and 24–h) three replicates were incubated, plus two blanks (no substrate), to calculate *in vitro* net gas production (GP) and the *in vitro* DM disappearance (IVDMD).

Each bottle was filled with 25 mL of the inoculum (39°C), gassed with CO_2 and sealed with a rubber stopper. Once the bottles were inoculated, they were incubated at 39°C for 24–h (Forma Series II 3110 Water–Jacketed CO_2 Incubator, Thermo Fisher Scientific, Waltham, USA) on an orbital shaker set at 90 oscillations/min (Heidolph Unimax, Germany). The incubations (runs) were repeated thrice during separate weeks, resulting in a total bottle number of 207 [(seven doses of PBE × three replicates + two blank bottles) × three sampling times × three runs].

Estimation of ruminal gas production, CH_4 and dry matter disappearance

Starting at 6–h of incubation, and then at 12 and 24–h, a sample of gas (15 mL) was collected with a syringe from each bottle and immediately transferred to a vacuumed exetainer (5.9 mL; Labco Ltd., Wycombe, Bucks, UK) and then analysed for CH_4 concentration by gas chromatography (Avila et al. 2011). The gas chromatograph (GC; Agilent 7890B, Agilent Technologies, Inc., Santa Clara, CA, USA) was equipped with a thermal conductivity detector (TCD) and a 30–m column (GS–CarbonPLOT, Agilent Technologies, Italy) using helium as carrier gas with a flow rate of 1.33 mL/min, and an isothermal oven temperature of 35°C. The injector and detector temperature were set to 185°C and 150°C, respectively. A subsample of gas (2 mL) was removed from each exetainer and injected manually into the GC. Methane gas of analytical quality (99.5%) was purchased from Linde (Santiago, Chile) to prepare the standards. Standards of CH_4 (15, 10, 7.5, 5.0, 2.5 and 1.0%) were prepared by diluting stock CH_4 gas with N gas at room temperature (≈22–24°C).

The total volume of gas produced in each bottle was measured using a water displacement apparatus according to Fedorak and Hrudey (1983). After gas sampling, the ANKOM F57 filter bags were removed from the bottles and washed with distilled water, followed by drying at 60°C for 24–h to estimate IVDMD (Avila et al. 2011).

Determination of culture pH and NH_3–N

The inoculum pH of every bottle was measured on a portable pH metre (Orion Star A121, Thermo Scientific, USA), and incubation fluid was sampled in a screw cap vial (2 mL; Biologix Research Company, USA) with trichloroacetic acid (150 µL; 0.65 w/v) to determine the NH_3–N concentration in a UV–VIS spectrophotometer (Merck, Spectroquant Pharo 300, Germany) at 625 nm. The cryotubes were stored at –20°C until analysis. At the beginning of each incubation, inoculum samples were collected and used to correct NH_3–N concentration (Avila et al. 2011).

Table 1. Ingredients (g/kg DM) and chemical composition (% of DM unless otherwise noted) of the substrates.

Ingredients	Substrate[a]						
	0.0%	0.3%	0.6%	0.9%	1.2%	1.5%	1.8%
Mixed hay	200	199	199	198	198	197	196
Soybean meal	200	199	199	198	198	197	196
Corn grain	600	599	596	595	592	591	590
Pine bark extract	–	3	6	9	12	15	18
Chemical composition							
Dry matter (% fresh weight)	88.2	86.9	85.2	83.3	82.0	81.6	81.0
Organic matter	95.6	95.5	95.6	95.6	95.5	95.7	95.5
Crude protein	21.3	19.0	18.4	18.2	19.0	18.8	19.3
Neutral detergent fiber	18.3	18.1	18.6	19.1	19.8	20.9	21.4
Acid detergent fiber	11.4	11.9	10.0	9.9	10.2	8.7	8.6
Hemicellulose	6.9	6.2	8.6	9.2	9.6	12.2	12.8
Metabolizable energy (Mcal/kg DM)	3.03	3.02	3.07	3.07	3.06	3.10	3.11

[a]Substrates had a polyphenolic extract from pine bark at different concentrations (% of dry matter basis).

Chemical analyses and calculated values

For substrates and PBE, DM (#934.01), ash (#942.05) and CP (#954.01) were conducted in accordance to the AOAC (1995) at the Animal Nutrition Laboratory, UdeC. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by Mertens (2002) and by procedure #973.18 of the AOAC (1995), respectively.

To estimate IVDMD kinetics, the calculated values were fitted using the non–linear Gompertz model (Lavrenčič et al. 1998; Equation 1):

$$y = B\exp[-C\exp(-At)] \quad (1)$$

where y = IVDMD (%) at t time; B = potentially degradable DM fraction (%); C = relative rate of degradation (/h); and A = describes a constant factor of microbial efficiency. The parameters B, C and A were used to calculate the first and second derivatives of the Gompertz model to obtain the degradation rate at the inflexion point (maximum degradation rate, MDR), and the time when 95% of the substrate is fermented (time of maximum degradation rate, TMDR), allowing a more comprehensive evaluation of small doses of PBE as feed supplement (Lavrenčič et al. 1998).

Organic matter (OM) in the substrates was estimated by the difference between DM and total ash, hemicellulose (HC) was estimated by the difference between the NDF and ADF, while DM digestibility (DMD) was estimated by Rohweder et al. (1978; Equation 2). Based on the value of DMD, digestible energy (DE) value was obtained using Fonnesbeck et al. (1984; Equation 3), and from the DE, metabolizable energy (ME) content was determined according to Equation 4 (NASEM 2016):

$$\text{DMD (\%)} = 88.9 - (0.779 \times \text{ADF}) \quad (2)$$

$$\text{DE (Mcal/kg DM)} = 0.27 + 0.0428 \times \text{DMD} \quad (3)$$

$$\text{ME (Mcal/kg DM)} = 0.82 \times \text{DE} \quad (4)$$

To estimate GP and CH_4 kinetics, recorded volumes were fitted using the non–linear Gompertz model (Schofield et al. 1994; Equation 5):

$$y = b\exp\{-\exp[1 - c(t - Lag)]\} \quad (5)$$

where y = gas (mL/0.5 g DM incubated) or CH_4 (mg/0.5 g DM incubated) production; b = asymptotic gas (mL/0.5 g DM incubated) or CH_4 (mg/0.5 g DM incubated) production; c = production rate (/h); Lag = initial delay (h) before gas or CH_4 production begins; and t = time of measurement. The half–life ($t_{1/2}$) is the time (h) taken for gas or CH_4 production to reach 50% of its b value. The average production rate (APR) was defined as the average gas (mL/0.5 g DM incubated) or CH_4 (mg/0.5 g DM incubated) production rate between the start of the incubation and the $t_{1/2}$ (García–Martínez et al. 2005).

To estimate fermentation efficiency, the partitioning factor (PF) at 24–h of incubation was determined as the ratio between degraded DM (mg) and the total GP (mL; Blümmel et al. 1997). Gas and CH_4 yields were estimated as the net gas (mL) or CH_4 (mg) volume at each sampling times (6, 12 or 24–h of incubation), divided by the corresponding g of degraded DM.

Estimation of microbial CP production (MCP) was based on Blümmel et al. (1997; Equation 6):

$$\text{MCP (mg/g DM)} = \text{mg DM degraded} - (\text{GP}_{24} \times 2.2\text{mg/mL}) \quad (6)$$

where GP_{24} = net gas production (mL/0.5 g DM) at 24–h; and 2.2 mg/mL is a stoichiometric coefficient of the amounts (mg) of C, H_2 and O_2 required for the production of volatile fatty acids associated with 1 mL of GP (Blümmel et al. 1997).

Short–chain fatty acid (SCFA) concentrations were calculated with equation 7 (Getachew et al. 2002):

$$\text{SCFA (mmol/200mg DM)} = 0.0222 \times \text{GP}_{24} - 0.00425 \quad (7)$$

where GP_{24} = production of net gas (mL/0.5 g DM) at 24–h.

Statistical analyses

Data were analyzed with Stata 14 statistical software (College Station, StataCorp LP, TX, USA). Shapiro–Wilk's and Levene's tests were used to verify the assumptions of normality and homogeneity of variances, respectively. All the data were analyzed in a randomized complete block design using the model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

where Y_{ij} is every observation of the PBE dose i on run j, μ is the general mean of observations, α_i is the fixed effect of the PBE dose (i = 0.0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8% DM basis), β_j is the random effect of the incubation run (j = 1, 2, 3) and ε_{ij} is the residual error. The results are presented as average values

with the standard error of mean. The averages were compared by Tukey's test, being statistically significant when $p < 0.05$ and considered a trend when $0.05 < p < 0.09$. In addition, polynomial contrasts were used to recognize linear and quadratic effects of increasing PBE concentrations.

Results

Chemical analyses of the treatments

The crude protein content in substrates was high (≥ 18.2 and ≤ 21.3% DM; Table 1), and as the polyphenolic PBE concentration increased, NDF and HC increased (18.3–21.4% DM and from 6.9–12.8% DM, respectively), whereas ADF and DM decreased (11.4–8.6% DM and from 88.2–81.0% fresh weight, respectively).

Dry matter disappearance and kinetics

The inclusion of increasing concentrations of PBE did not affect the IVDMD at 6–h ($p = 0.865$), but resulted in a linear reduction at 12 ($p = 0.001$) and 24–h ($p = 0.003$), being from 3 to 5% lower than control at 12–h ($p = 0.004$) with the highest doses of PBE (1.5 and 1.8%), and a 6% lower at 24–h ($p = 0.032$) with a 1.8% PBE (Table 2). The potentially degradable DM fraction (*B*) also decreased linearly ($p = 0.002$) with increasing PBE concentrations, being in average a 4% lower with a 1.5 and 1.8% PBE ($p = 0.023$), whereas relative rate of degradation (*C*), microbial efficiency (*A*), TMDR and MDR were unaffected ($p \geq 0.507$) by the extract.

In vitro ruminal gas production and kinetics

Net gas production at 6–h was unaffected by PBE ($p = 0.730$; Table 3), but at 12 and 24–h it was decreased linearly ($p < 0.001$), being lower with a 1.5 and 1.8% PBE at 12–h ($p = 0.011$), and with 1.2, 1.5 and 1.8% PBE at 24–h ($p < 0.001$). Total GP at 6 and 12–h was unaffected by treatments ($p = 0.971$ and $p = 0.432$, respectively). However, increasing concentrations of PBE linearly increased *in vitro* GP at 12 ($p = 0.035$) and 24–h ($p = 0.006$), and trended ($p = 0.073$) to a higher GP with 1.5 and 1.8% PBE at 24–h than control. These results agree with those of gas yield (GY), which increased linearly at 12 ($p = 0.017$) and 24–h ($p = 0.004$), with a trend ($p = 0.067$) to a higher GY with 1.5 and 1.8% PBE at 24–h whereas asymptotic GP (*b*) also increased linearly ($p = 0.007$) with PBE addition, trending to be higher with 1.5 and 1.8% ($p = 0.072$). Gas production rate (*c*), lag time, $t_{1/2}$ and the APR were not affected by PBE ($p \geq 0.327$).

Ruminal CH_4 production and kinetics

Methane proportion of net gas (Table 4) decreased linearly at 6–h of incubation ($p = 0.029$), and trended to decrease at 12 and 24–h ($p = 0.060$ and $p = 0.088$, respectively). Likewise, the *in vitro* CH_4 production decreased linearly at 6–h of incubation ($p = 0.001$), achieving the lowest productions ($p = 0.016$) with 1.5 and 1.8% PBE as compared to control (1.1 vs 1.6 mg/0.5 g DM incubated). However, at 12 and 24–h there was no effect ($p = 0.900$ and $p = 0.914$, respectively) of PBE supplementation. Methane yield was unaffected at 6 ($p = 0.994$), 12 ($p = 0.619$) and 24–h ($p = 0.999$), whereas CH_4 production parameters, lag time increased linearly ($p = 0.005$), being higher ($p = 0.037$) with the highest doses of PBE (1.5 and 1.8%), whereas asymptotic CH_4 production (*b*), CH_4 production rate (*c*), $t_{1/2}$ and the APR were unaffected by PBE ($p \geq 0.511$).

In vitro fermentation

Increasing PBE concentrations linearly increased pH ($p = 0.002$) and SCFA ($p < 0.001$), being higher ($p = 0.007$) in the dietary substrates supplemented with 1.5 and 1.8% PBE (Table 5). Microbial Crude Protein ($p < 0.001$) and the PF_{24} ($p = 0.001$) were linearly decreased, being both parameters lower ($p < 0.005$) with the highest PBE supplementation (1.5 and 1.8%). The *in vitro* NH_3–N concentrations linearly decreased at 24–h ($p < 0.001$), and were reduced by 31% as compared to control ($p = 0.001$) with the highest doses of PBE (1.5 and 1.8%).

Table 2. Effect of a polyphenolic extract from pine bark (PBE) at different concentrations (% of DM basis) as feed additive in *in vitro* DM disappearance (IVDMD) and kinetics.

Item	PBE (%)	IVDMD parameters[a]					IVDMD (%)		
		B	*C*	*A*	TMDR	MDR	6–h	12–h	24–h
Substrate	0.0	63.3^{B}	0.89	0.23	5.0	51.1	35.3	49.2^{B}	63.7^{B}
	0.3	60.9^{AB}	0.85	0.22	4.7	53.9	34.8	46.2^{AB}	61.0^{AB}
	0.6	62.8^{AB}	0.84	0.19	4.3	59.9	34.4	47.2^{AB}	63.1^{AB}
	0.9	62.2^{AB}	0.87	0.20	4.5	57.4	34.2	47.4^{AB}	62.1^{AB}
	1.2	60.5^{AB}	0.84	0.20	4.5	57.2	33.9	46.4^{AB}	61.6^{AB}
	1.5	59.2^{A}	0.82	0.21	4.4	52.8	34.7	43.8^{A}	59.8^{AB}
	1.8	59.6^{A}	0.83	0.22	4.7	53.4	33.7	45.8^{A}	58.2^{A}
Pooled SEM[b]		0.88	0.063	0.008	0.37	6.00	0.90	0.93	1.15
p value[c]									
T		0.023	0.969	0.507	0.745	0.910	0.865	0.004	0.032
L		0.002	0.485	0.568	0.427	0.943	0.176	0.001	0.003
Q		0.873	0.846	0.068	0.142	0.265	0.654	0.512	0.234

[A–B]Different letters in same column indicate significant differences ($p < 0.05$).
[a]*B* = potentially degradable dry matter fraction (%); *C* = relative rate of degradation (/h); *A* = constant factor of the microbial efficiency; TMDR = time of maximum degradation rate (h); MDR = maximum degradation rate (%/h).
[b]SEM = Standard error of mean.
[c]Probability of differences between treatments (T), or of a linear (L) or quadratic (Q) effect by PBE concentration.

Table 3. Effect of a polyphenolic extract from pine bark (PBE) at different concentrations (% of DM basis) as an additive for feed in *in vitro* gas output and kinetics parameters.

Item	PBE (%)	Gas production parameters[a]					Net gas (mL)			Gas production (mL/0.5 g DM incubated)			Gas yield (mL/0.5 g DM degraded)		
		B	*c*	*Lag*	$t_{1/2}$	APR	6–h	12–h	24–h	6–h	12–h	24–h	6–h	12–h	24–h
Substrate	0.0	134.9	0.20	3.1	6.0	10.2	12.8	38.2^{B}	59.5^{C}	28.2	82.6	130.8	81.5	172.2	204.9
	0.3	134.5	0.18	2.9	5.9	10.3	12.6	36.4^{AB}	58.7^{BC}	29.8	83.0	132.5	82.8	174.1	206.8
	0.6	144.7	0.19	3.2	5.9	10.5	12.2	36.2^{AB}	58.0^{BC}	28.3	83.7	132.8	83.4	177.0	214.7
	0.9	140.8	0.20	3.1	5.9	10.3	12.5	36.1^{AB}	57.5^{BC}	28.2	85.1	134.7	84.5	180.1	217.3
	1.2	139.4	0.19	2.6	5.8	10.6	12.4	36.0^{AB}	55.3^{AB}	29.6	86.5	133.6	85.9	181.5	217.6
	1.5	149.1	0.19	3.1	6.0	10.5	12.3	34.8^{A}	56.1^{AB}	28.8	85.5	139.6	80.9	180.6	230.7
	1.8	147.8	0.18	3.3	6.2	10.3	11.5	34.8^{A}	54.0^{A}	28.3	86.1	136.1	79.5	182.4	221.8
Pooled SEM[b]		3.40	0.014	0.36	0.15	0.15	0.69	0.78	0.84	1.95	1.83	2.18	4.70	4.53	7.17
p value[c]															
T		0.072	0.901	0.748	0.424	0.418	0.730	0.011	< 0.001	0.971	0.432	0.073	0.945	0.328	0.067
L		0.007	0.448	0.822	0.327	0.336	0.134	< 0.001	< 0.001	0.958	0.035	0.006	0.748	0.017	0.004
Q		0.953	0.724	0.625	0.119	0.137	0.604	0.476	0.746	0.734	0.596	0.841	0.299	0.505	0.534

[A–C]Different letters in same column indicate significant differences ($p < 0.05$).

[a]*b* = asymptotic production (mL gas/0.5 g DM incubated); *c* = rate of gas production (/h); *Lag* = initial delay before gas production begins (h); $t_{1/2}$ = half–life (h); APR = average production rate (mL/g DM incubated per h).

[b]SEM = Standard error of mean.

[c]Probability of differences between treatments (T), or of a linear (L) or quadratic (Q) effect by PBE concentration.

Table 4. Effect of a polyphenolic extract from pine bark (PBE) at different concentrations (% of DM basis) as an additive for feed in *in vitro* CH_4 output and kinetics parameters.

Item	PBE (%)	CH_4 production parameters[a]					CH_4 proportion of net gas (%)			CH_4 production (mg/0.5 g DM incubated)			CH_4 yield (mg/0.5 g DM degraded)		
		b	*c*	*Lag*	$t_{1/2}$	APR	6–h	12–h	24–h	6–h	12–h	24–h	6–h	12–h	24–h
Substrate	0.0	25.0	0.15	6.9^{A}	10.1	1.1	4.1	8.6	12.4	1.6^{B}	8.8	21.7	4.6	17.9	34.7
	0.3	27.7	0.13	7.1^{AB}	10.5	1.2	3.6	8.7	12.7	1.3^{AB}	8.3	22.7	4.1	17.8	35.1
	0.6	27.6	0.14	7.1^{AB}	10.5	1.2	3.8	8.5	12.6	1.5^{AB}	8.2	22.5	4.5	16.2	35.1
	0.9	27.0	0.14	7.1^{AB}	10.5	1.2	3.5	8.3	12.8	1.2^{AB}	8.2	22.4	4.4	15.5	35.8
	1.2	26.6	0.14	7.2^{AB}	10.5	1.3	3.3	8.1	13.0	1.3^{AB}	8.1	22.2	4.4	16.0	35.9
	1.5	25.8	0.15	7.4^{B}	10.4	1.1	3.4	7.8	12.9	1.1^{A}	8.1	21.9	4.1	15.4	35.5
	1.8	25.2	0.15	7.4^{B}	10.7	1.3	3.0	7.7	13.3	1.1^{A}	8.1	22.1	4.1	15.3	35.9
Pooled SEM[b]		2.07	0.009	0.12	0.42	0.07	0.39	0.50	0.41	0.12	0.53	0.95	0.71	1.38	2.97
p Value[c]															
T		0.645	0.807	0.037	0.943	0.511	0.420	0.676	0.712	0.016	0.900	0.914	0.994	0.619	0.999
L		0.621	0.402	0.005	0.390	0.176	0.029	0.060	0.088	0.001	0.252	0.830	0.679	0.090	0.731
Q		0.251	0.527	0.629	0.809	0.981	0.952	0.712	0.793	0.615	0.460	0.442	0.874	0.498	0.910

[A–B]Different letters in same column indicate significant differences ($p < 0.05$).
[a]*b* = asymptotic production (mg methane/0.5 g DM incubated); *c* = rate of methane production (/h); *Lag* = initial delay before methane production begins (h); $t_{1/2}$ = half–life (h); APR = average production rate (mg/g DM incubated per h).
[b]SEM = Standard error of mean.
[c]Probability of differences between treatments (T), or of a linear (L) or quadratic (Q) effect by PBE concentration.

Discussion

As the PBE concentration increased in the substrate, DM decreased numerically given the aqueous state (38.0% DM) of the extract. Both NDF and ADF substrates values were altered by PBE supplementation. If both had been increased, it could have indicated that the increase, although numerical, may be associated to the NDF and ADF contents of the extract (48.6 and 64.9% DM, respectively). However, NDF increased and ADF decreased, which could be related to the influence of condensed tannins in the PBE which form complexes with fibre and hinder the use of conventional detergent method of fibre analysis (Guglielmelli et al. 2011; Vera et al. 2018). The substrates were high in CP to simulate the use of spring forages and concentrate diets with high protein values, of which an important fraction is lost as NH_3–N (Guglielmelli et al. 2011).

Table 5. Effect of a polyphenolic extract from pine bark (PBE) at different concentrations (% of DM basis) as feed additive in *in vitro* ruminal fermentation profile[a], after 24–h of incubation.

Item	PBE (%)	pH	NH_3–N (mg/dL)	PF_{24} (mg DM degraded/mL)	SCFA (mmol/g DM)	MCP (mg/g DM)
Substrate	0.0	6.3	9.7^{B}	4.1^{B}	2.8^{A}	154.4^{B}
	0.3	6.3	9.6^{B}	3.9^{AB}	2.8^{A}	147.5^{AB}
	0.6	6.4	9.5^{B}	3.9^{AB}	2.9^{AB}	142.2^{AB}
	0.9	6.4	7.9^{AB}	3.9^{AB}	3.0^{AB}	138.8^{AB}
	1.2	6.4	7.4^{AB}	3.9^{AB}	3.0^{AB}	133.5^{AB}
	1.5	6.4	6.7^{A}	3.8^{A}	3.1^{B}	122.7^{A}
	1.8	6.4	6.7^{A}	3.8^{A}	3.1^{B}	123.7^{A}
Pooled SEM[b]		0.03	0.63	0.06	0.05	5.90
p value[c]						
T		0.074	0.001	0.014	0.007	0.001
L		0.002	< 0.001	0.001	< 0.001	< 0.001
Q		0.845	0.918	0.813	0.825	0.857

[A–B]Different letters in same column indicate significant differences ($p < 0.05$).
[a]pH = potential hydrogen; NH_3–N = ammonia nitrogen; PF_{24} = partitioning factor at 24–h of incubation; SCFA = short–chain fatty acids; MC*P* = microbial crude protein production.
[b]SEM = Standard error of mean.
[c]Probability of differences between treatments (T), or of a linear (L) or quadratic (Q) effect by PBE concentration.

The linear decrease in the potentially degradable DM fraction (*B*) and the IVDMD by increasing polyphenolic PBE concentrations can be attributed to reduced fibre digestibility by the formation of complexes of tannins and lignocellulose, or by the inhibition of cellulolytic microorganisms or their enzymes (Deaville et al. 2010; Vasta et al. 2019), as reported by Ahnert et al. (2015) in heifers receiving increasing concentrations of a quebracho tannin extract or by Brutti et al. (2019), who used a mixture of chestnut and quebracho tannins under *in vitro* conditions.

The increased asymptotic GP (*b*) was negatively correlated with ADF in the substrate, concurring with Kafilzadeh and Heidary (2013), who reported that with increasing incubation time, the medium conditions vary by releasing cell wall components that could affect rumen microbial activity. It is possible that as PBE increased in the substrate, the numerical decrease of the ADF improved the microbial activity, increasing *b*, through favourable environmental conditions as incubation time progressed. After 12 and 24–h of batch incubation, GP and GY increased with PBE inclusion, suggesting that it contains fermentable compounds. Gas production depends on the available nutrients for microorganisms (Elghandour et al. 2016) and therefore, it is possible that PBE provided an additional nutritional contribution to the inoculum microorganisms. Flavonoids, such as quercetin, are metabolized in the rumen by hydrolysis of the glycoside moiety and cleavage of the heterocyclic ring, producing di– and monohydroxyphenolics, phloroglucinol and SCFA such as acetate and butyrate (McSweeney et al. 2002). Alternatively, according to Jiménez–Peralta et al. (2011), some rumen bacteria can metabolize various phenolic compounds cross–linking polysaccharides and lignin, thus increasing fermentation and GP. However, this is unlikely to have occurred in this study, as IVDMD was not increased. Moreover, the most common response to tannin or polyphenol inclusion in ruminant diets is a decrease in *in vitro* GP (Rira et al. 2015; Brutti et al. 2019).

At 6–h of incubation, a 1.8% PBE in DM reduced the CH_4 production (–26.7%) but not at 12 and 24–h, concurring with previous studies (Oliveira et al. 2007; Szczechowiak et al. 2016)

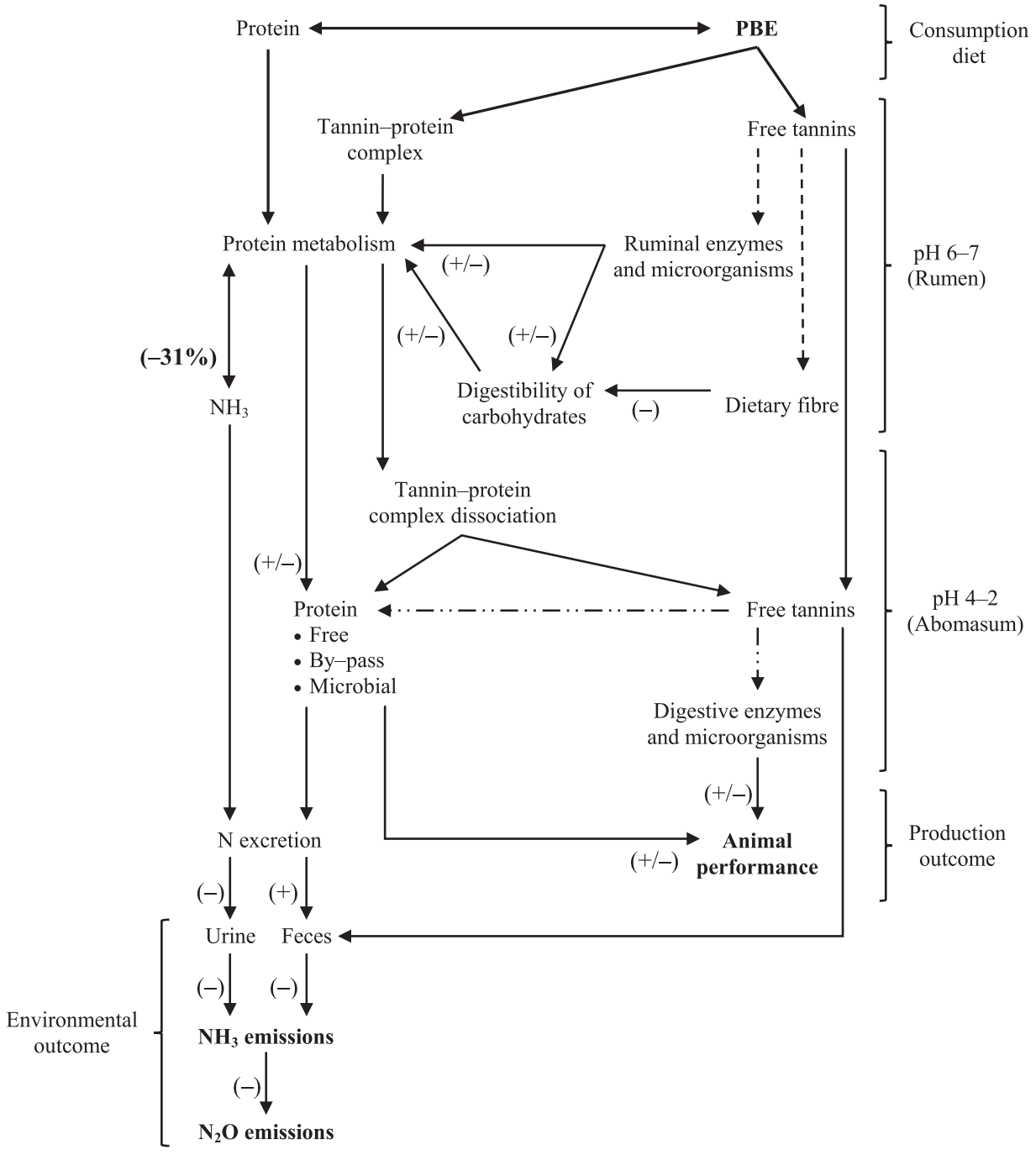

Figure 1. Potential environmental and productive effects by the supplementation of an extract from pine bark (PBE) on diet. Binding capacity (– →) and possibility of union (– →); signs '+' and '–' indicate increase and decrease, respectively.

where the tannin content in the diet did not reduce CH_4 production. However, mitigation of CH_4 production by flavonoids has been reported previously in several reviews (Patra and Saxena 2010; Vasta et al. 2019). In addition, leaves of different tannin-rich tropical trees suppressed CH_4 production in different magnitudes in *in vitro* (Bhatta et al. 2015) and *in vivo* conditions (Malik et al. 2017). Because the CH_4 lag time was increased by PBE supplementation, while the 6–h IVDMD and GP were unaffected, along with the fact that the SCFA profile could not be determined in this study, we suggest that the PBE polyphenols reduce CH_4 production in the first hours of incubation by a delayed microbial colonization and growth rate (Firkins et al. 1998), an initial methanogen inhibition (Elghandour et al. 2016), or by a change in the SCFA profile, since fermentation to propionate increases hydrogen consumption, whereas acetate formation produces hydrogen (Patra and Saxena 2010; Vasta et al. 2019).

The concentrations of NH_3–N decreased when inclusions of PBE reached 1.5% (–31% at 24–h incubation). These concentrations were never lower than 5 mg/dL of NH_3, which is the minimum level required for adequate DM digestion (Junior et al. 2017). *In vitro* NH_3–N concentration represents the balance between degraded dietary protein, absorption through the rumen wall and use for microbial protein synthesis

(Hariadi and Santoso 2010). Decreased NH_3–N ls can be attributed to an NH_3 inhibition by increasing doses of polyphenols in supplemented substrates, which can affect the deamination process (Purba et al. 2020b), decrease dietary protein degradation by inhibiting protease activity (Brutti et al. 2019) and/or create a pH–dependent (6.0–6.5) tannin–protein complex. In the abomasum, at pH 2.0, the complex dissociates, allowing peptidase action (Junior et al. 2017). The reduction of rumen CP degradation leads to a more efficient dietary protein use through the generation of 'by–pass protein', which may result in improved animal performance and decreased urea N excretion. However, the N losses via faeces (more resilient to environmental loss) should be slightly increased (Deaville et al. 2010). Despite the above, tannin supplementation lowers urinary N excretion (Figure 1), but has had detrimental effects on animal performance (Aguerre et al. 2016). This suggests that by decreasing the protein degradation of the diet, the MCP could be affected by tannins via enzyme inhibition, affecting the viability of essential metal ions, and/or by changes in the bacterial cell wall, which would compensate for the increase of by–pass protein (Ahnert et al. 2015).

The decreased NH_3–N concentration concurs with other studies conducted *in vitro*, e.g. by Castro–Montoya et al. (2018) with quebracho (*S. lorentzii*) tannins, Purba et al. (2020b) with betel (*Piper betle*) powder, an abundant source of polyphenols; as well as *in vivo*, e.g. by Hatami et al. (2018), who fed growing lambs with 80 g/kg DM of pomegranate (*Punica granatum*) marc (16.8 g TT/kg DM) and reported reduced ruminal NH_3–N concentration (–40%) and N excretion via urine (–27%). Supplementing Holstein cows with a mixture of quebracho and chestnut tannin (0.45% DM) also reduced ruminal NH_3–N concentration (–9%) and N excretion via urine (Aguerre et al. 2016).

The average pH value varied from 6.3–6.4 in 24–h; an optimal range of 6.7 ± 0.5 is required to maintain normal cellulolytic activities, and pH values above 6.0 are required for microbial protein synthesis (Hariadi and Santoso 2010). Flavonoids are the main PBE components and can prevent pH decrease by having a direct buffer effect or by increasing the activity of lactate–consuming bacteria (Balcells et al. 2012; Goto et al. 2016).

The calculated SCFA increase with PBE addition is related to a GP increase, since there is a good association between calculated SCFA and *in vitro* GP; the degraded substrate in a closed *in vitro* gas system is converted into gases, SCFA, water and microbial mass (Makkar 2005). Higher SCFA concentrations reflect a greater amount of fermented substrate by rumen microorganisms (Purba et al. 2020c), however, as the IVDMD decreased by PBE inclusion in the substrate, the increasing concentration of SCFA in supplemented substrates can be attributed to the presence of some flavonoids in the extract, as quercetin, which have been shown to increase SCFA concentration (Purba et al. 2020b). This increase in SCFA can be beneficial, as these are the main end products of fermentation and represent the major supply of energy for ruminants (Salem et al. 2015). By contrast, both the PF_{24} and the calculated MCP decreased with increasing PBE doses. A decrease in PF indicates that less substrate was converted into microbial biomass (Elghandour et al. 2016), possibly due to the tannins presents in the PBE that form tannin–protein or –fibre complexes (Jiménez–Peralta et al. 2011); in addition, CT can bind to ruminal microorganisms or their enzymes, inhibiting their growth (Castro–Montoya et al. 2018).

Our results indicate that the supplementation of PBE at a concentration of 1.5% DM basis in high–concentrate diets could decrease N excretion in ruminants, given the NH_3–N concentration reduction (–31%). In addition, PBE can delay CH_4 production (–31%), but this delay is offset after 12 or 24–h. This suggests that PBE supplementation in ruminant diets has the potential to contribute to improve sustainability of environmentally friendly animal production systems. However, before it can be used in *in vivo* conditions and to validate its effects, it is necessary to perform long–term incubations (Rumen Simulation Technique, RUSITEC) and to clarify the effect of supplementing with 1.5% PBE on rumen microorganisms.

Conclusion

Supplementing high–concentrate diets with least 1.5% DM of a polyphenolic extract from pine bark (PBE) reduces CH_4 production at 6–h of incubation, but not at 24–h. However, at 24–h incubations NH_3–N concentration can be reduced by 31% with a slight reduction in digestibility at 1.8% PBE inclusion. Our results warrant future research in long term incubations and under *in vivo* conditions to confirm PBE potential contribution to livestock systems sustainability.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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