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Effects of vanillin, quillaja saponin, and essential oils on in vitro fermentation and protein-degrading microorganisms of the rumen

Amlan K. Patra · Zhongtang Yu

Abstract This study investigated the effects of vanillin on methanogenesis and rumen fermentation, and the responses of ruminal protein-degrading bacteria to vanillin (at concentrations of 0, 0.76 and 1.52 g/L), essential oils (clove oil, 1 g/L; origanum oil, 0.50 g/L, and peppermint oil, 1 g/L), and quillaja saponin (at concentration of 0 and 6 g/L) in vitro. Methane production, degradabilities of feed substrate, and ammonia concentration decreased linearly with increasing doses of vanillin. Concentration of total volatile fatty acids also decreased, whereas proportion of butyrate tended to increase linearly with increasing doses of vanillin. Protozoa population decreased, but abundances of *Ruminococcus flavefaciens*, *Prevotella bryantii*, *Butyrivibrio fibrisolvens*, *Prevotella ruminicola, Clostridium aminophilum*, and *Ruminobacter amylophilus* increased with increasing doses of vanillin. Origanum and clove oils resulted in lower ammonia concentrations compared to control and peppermint oil. All the tested essential oils decreased abundances of protozoa, *Selenomonas ruminantium*, *R. amylophilus*, *P. ruminicola* and *P. bryantii*, with the largest decrease resulted from origanum oil followed by clove oil and peppermint oil. The abundances of *Megasphaera elsdenii*, *C. aminophilum*, and *Clostridium sticklandii* were deceased by origanum oil while that of *B. fibrisolvens* was lowered by both origanum and clove oils. Saponin decreased ammonia concentration and protozoal population, but increased the abundances of *S. ruminantium*, *R. amylophilus*, *P. ruminicola*, and *P. bryantii*, though the magnitude was small (less than one log unit). The results suggest that reduction of ammonia production by vanillin and saponin may not be caused by direct inhibition of major known proteolytic bacteria, and essential oils can have different inhibitory effects on different proteolytic bacteria, resulting in varying reduction in ammonia production.

Keywords Ammonia · Essential oil · Proteolytic bacteria · Quillaja saponin · Rumen · Vanillin

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Introduction

Presently, livestock farming faces challenges in reducing its environmental impact while sustaining economic viability due to increasing cost and shortage of feeds. There is an urgent need to meet these challenges by decreasing methane and nitrous oxide emissions and nitrogen excretion, reducing overuse of antibiotic feed additives, while improving utilization of nutrients. Livestock contribute 12–18 % to the global anthropogenic greenhouse gas emissions depending upon emission attributes (FAO 2006; Westhoek et al. 2011), and account for about 37 % of the total anthropogenic methane and 65 % of global anthropogenic nitrous oxide (FAO 2006). Of the total anthropogenic methane (5.9×10^9 tonnes CO_2 equivalent per year), 30 % arises from enteric methane emission, mostly from fermentation of feeds in the rumen (FAO 2006). Nitrogen excretion by livestock and nitrous oxide emission from manure are other major concerns, both of which contribute to air and ground water pollution. Excessive protein degradation and amino acid deamination in the rumen are nutritionally wasteful processes because more ammonia is produced than rumen microorganisms can utilize (Wallace et al. 1997; Firkins et al. 2007). Excess ammonia is absorbed by the animals and is excreted mainly as urinary urea. Ruminant nitrogen excretion is a major source of environmental pollution (FAO 2006), and as much as 60–90 % of the feed nitrogen can be excreted depending upon

animal species and physiological stages of animals (Flachowsky and Lebzien 2006). Because ammonia can be oxidized by nitrifying bacteria, livestock operation can lead to groundwater pollution with nitrate and nitrite (Philips et al. 2002). Of course, low nitrogen utilization efficiency results in increased cost of ruminant farming. Ruminant nutritional research has, therefore, been focused on decreasing ruminal protein degradation and ammoniagenesis.

In recent years, natural plant products containing essential oils (EOs), saponins, or tannins have been widely evaluated as non-antibiotic feed additives to mitigate methane emission, suppress protein degradation in rumen, increase feed utilization efficiency, and improve characteristics of rumen fermentation (Patra and Saxena 2009a; Calsamiglia et al. 2007; Patra 2011a,b; Patra and Saxena 2010). Castillejos et al. (2006) reported that vanillin can modulate rumen volatile fatty acid (VFA) characteristics, but its effects on other aspects of rumen fermentation and rumen microbial populations remain uninvestigated. Recently, we investigated the effects of EO and saponins on rumen fermentation, methanogenesis, and rumen bacterial and archaeal diversity using various molecular tools (Patra and Yu 2012; Patra et al. 2012). In these studies, EO was found effective in inhibiting methane production and modulating rumen fermentation depending upon type and dose of EO. Saponins also altered rumen fermentation and microbial diversity. However, effects of EO and saponins on various protein-degrading and amino acid-deaminating microbes remain poorly understood. The objective of this study was, hence, to investigate the effects of vanillin on methane production, fermentation characteristics, abundance of archaea, protozoa, and cellulolytic and protein-degrading bacterial population using real-time PCR. The effects of EO and saponins on protein-degrading microorganisms were also investigated.

Materials and methods

Plant secondary compounds

In experiment 1, vanillin (Sigma-Aldrich, St. Louis, MO, USA) was used at two doses (0.76 and 1.52 g/L). In experiment 2, clove oil (CLO, *Eugenia* spp.), origanum oil (ORO, *Thymus capitatus* L. Hoffmanns & Link), and peppermint oil (PEO, *Mentha piperita* L.) (Sigma-Aldrich) were used at 1.0, 0.5, and 1.0 g/L, respectively, while quillaja saponins (from the bark of *Quillaja saponaria* Molina plants; Sigma-Aldrich) was used at 0.6 g/L. The doses of EO and saponin were used as they were found to decrease ammonia concentrations in previous studies (Patra and Yu 2012; Patra et al. 2012). In both experiments, controls were included in parallel that contained none of the above phytochemicals.

Ruminal inoculum and in vitro incubations

The ruminal inoculum for in vitro incubations was collected from two fistulated lactating Jersey cows at approximately 9 h postmorning feeding. The total mixed ration (TMR) fed to the cows was composed (percent dry matter basis) of corn silage (33 %), alfalfa and mixed grass hay (8.5 %), and a concentrate mixture (58.5 %). The cows were offered the TMR twice a day at 6 AM and 6 PM. The rumen fluid from each cow was obtained after squeezing the rumen content into a sterilized glass bottle (500 mL) leaving no headspace in the bottle. The fresh rumen fluid samples were brought to the laboratory within 10 min to minimize effect on microbial viability. The rumen fluid samples were then strained through four layers of cheesecloth in an anaerobic chamber (Coy Laboratory Products Inc., USA) and combined together in an equal volume.

The in vitro incubation was carried out in 120-mL serum bottles in triplicates for each treatment (including controls). The in vitro buffered medium (Menke and Steingass 1988) was prepared anaerobically as described earlier (Patra et al. 2010; Patra and Yu 2012). Inside the anaerobic chamber, 30 mL of the anaerobic medium and 10 mL of rumen fluid were dispensed into each serum bottles containing 400 mg of the ground feed substrate. The feed substrate consisted of (50:50 ratio) alfalfa hay and a dairy concentrate mixture that consists mainly of ground corn (33.2 %), soybean meal (14.2 %), AminoPlus® (15.5 %), distillers grains (19.8 %), and wheat middlings (11.3 %). After sealing with butyl rubbers plus crimped aluminum seals, the serum bottles were incubated at 39 °C for 24 h in a water bath with intermittent manual shaking.

Sampling

After 24 h of incubation, gas pressure in the bottle was measured using a manometer (Traceable®; Fisher Scientific, USA) to determine total gas production. Then, headspace gas was collected into glass tubes filled with distilled water by displacement for methane analysis. One milliliter of each in vitro culture content was collected in microcentrifuge tubes for microbial analysis. Then, pH values of the in vitro cultures were immediately measured using a pH meter (Fisher Scientific, USA). The remaining content was filtered through filter bags (ANKOM Technology, USA; pore size of 50 μm) to determine degradability of the feed substrate. The filtrates were sampled in microcentrifuge tubes for VFA and ammonia analyses. All the samples were stored at −20 °C until further analyses.

Rumen fermentation measurement

The concentrations of methane in the gas samples were determined using a gas chromatograph (HP 5890 Series,

Agilent Technologies, USA) equipped with a thermal conductivity detector and a HP-PLOT Q capillary column coated with porous polymer particles made of divinylbenzene and ethylvinylbenzene (Agilent Technologies Inc, USA). The VFA concentrations in the in vitro cultures were analyzed also by gas chromatography (HP 5890 series, Agilent Technologies, USA) fitted with a flame ionization detector and a Chromosorb W AW packed glass column (Sigma-Aldrich, USA). The concentrations of ammonia were determined colorometrically (Chaney and Marbach 1962). The degradabilities of dry matter (DM) and neutral detergent fiber (NDF) were determined as described earlier (Patra and Yu 20125).

DNA extraction

Metagenomic DNA was extracted following the procedure described by Yu and Morrison (2004). The DNA quality was evaluated using agarose gel (1 %) electrophoresis, and DNA yield was quantified using a Quant-iT dsDNA Broad Range Assay kit (Invitrogen Corporation, Carlsbad, CA, USA) in a Stratagene Mx3000p machine (La Jolla, CA, USA). The DNA samples were stored at −20 °C until analysis.

Quantitative real-time PCR analyses

The population sizes of archaea, protozoa and select bacterial species were quantified using SYBR Green-based quantitative real-time PCR (qPCR) using a Stratagene Mx3000p system (La Jolla, CA, USA). The primers and PCR conditions used are shown in Table 1. A sample-derived qPCR standard was prepared for each target group using the respective specific PCR primer set and a composite DNA sample that was prepared by pooling an equal amount of all the metagenomic DNA samples (Yu et al. 2005). The 50 μL PCR amplification reaction mixture contained 1× PCR buffer, 1.75 mM (3.5 mM for *M. elsdenii* and *Clostridium sticklandii*) MgCl$_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 0.67 mg/L bovine serum albumin, 1.25 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), and 1 μL (50 ng; 100 ng for *C. sticklandii*) of the pooled DNA. Each of the standards were then purified using a PCR Purification kit (Qiagen, USA) and quantified using a Quant-iT dsDNA Broad Range Assay kit (Invitrogen). For each of the standards, 16S rRNA (*rrs*) gene copy-number concentration was calculated based on the length of the PCR products and its mass concentration (Yu et al. 2005). Tenfold serial dilutions were made in Tris-EDTA (TE) buffer prior to qPCR assays. The qPCR was done as described previously (Yu et al. 2005), and fluorescence signals resulting from possible primer-dimers was excluded using the fluorescence signal acquired at 86 °C, at which primer–dimers were completely denatured but not the expected amplicons, as

verified by melting curve analysis (Yu et al. 2005). To minimize variations, the qPCR assay for each species or group was performed in triplicates for both the standards and the metagenomic DNA samples using the same master mix and the same PCR plate. The absolute abundances were expressed as *rrs* gene copies/milliliter of culture samples.

Statistical analysis

The data on rumen fermentation characteristics and abundances (log *rrs* gene copies/milliliter samples) of quantified ruminal microorganisms were analyzed using the PROC MIXED procedure of SAS (2001). Orthogonal polynomial contrasts were used to examine linear and quadratic responses to the increasing doses of vanillin. Significance was declared at $P \leq 0.05$, whereas $0.05 < P \leq 0.10$ values were considered to be a trend. When a significant effect of treatments was detected, all pairwise significant differences between treatment means were separated by Fisher's protected least significant difference using SAS (2001).

Results

Effects of vanillin on total gas and methane production, substrate degradability, and ammonia concentrations

Total gas production by the mixed in vitro rumen cultures decreased quadratically with increasing doses of vanillin, while methane production, degradabilities of DM and NDF, pH, and ammonia concentration in the cultures decreased linearly in response to increasing doses of vanillin (Table 2). Total gas and methane production and DM degradability were significantly lower only at the high vanillin dose (10 mM) than the control. Compared to the control, NDF degradability was significantly lower at the high vanillin dose, but only numerically lower at the low vanillin dose (5 mM). Ammonia concentration was the lowest at the high dose of vanillin, followed by low dose and then the control.

Effects of vanillin on volatile fatty acid concentrations

Total VFA concentration, molar proportions of total branched-chain volatile fatty acid (BCVFA), valerate, and isovalerate decreased linearly, whereas molar proportion of butyrate increased linearly with increasing doses of vanillin in the mixed in vitro rumen cultures (Table 3). Molar proportions of acetate, propionate and isobutyrate, and acetate/propionate ratio were not affected by vanillin. Total VFA and molar proportion of valerate were significantly lower for the high vanillin dose than the low vanillin dose

Table 1 Primers used for real-time PCR quantification of archaea, protozoa, and rumen bacterial species

Target organisms	Primers	Sequences (5′→3′)[a]	Annealing temperature (°C)	Amplicon length (bp)	References
Archaea	ARC787f ARC1059r	ATT AGA TAC CCS BGT AGT CC GCC ATG CAC CWC CTC T	56	272	Yu et al. 2005
Protozoa	P.SSU-54f P.SSU-1747r	CAY GTC TAA GTA TAA ATA ACT AC CTCTAGGTGATWWGRTTTAC	55	1,693	Sylvester et al. 2004
	316f 539r	GCT TTC GWT GGT AGT GTA TT CTT GCC CTC YAA TCG TWC T	54	223	
F. succinogenes	Fs219f Fs654r	GGT ATG GGA TGA GCT TGC GCC TGC CCC TGA ACT ATC	63	446	Koike and Kobayashi 2001
R. flavefaciens	Rf154f Rf425r	TCT GGA AAC GGA TGG TA CCT TTA AGA CAG GAG TTT ACA A	55	295	Koike and Kobayashi 2001
R. albus	Ra1281f Ra1439r	CCC TAA AAG CAG TCT TAG TTC G CCT CCT TGC GGT TAG AAC A	55	175	Koike and Kobayashi 2001
R. amylophilus	Ram-F Ram-R	CAA CCA GTC GCA TTC AGA CAC TAC TCA TGG CAA CAT	57	642	Tajima et al. 2001
P. ruminicola	P.rumi-F P.rumi-R	GGT TAT CTT GAG TGA GTT CTG ATG GCA ACT AAA GAA	53	485	Bekele et al. 2010
P. bryantii	P.brya-F P.brya-F	ACT GCA GCG CGA ACT GTC AGA ACC TTA CGG TGG CAG TGT CTC	67	540	Bekele et al. 2010
B. fibrisolvens	ButFib2F ButFib2R	ACC GCA TAA GCG CAC GGA CGG GTC CAT CTT GTA CCG ATA AAT	62	65	Stevenson and Weimer 2007
S. bovis	S. bovis-F S. bovis-R	CTA ATA CCG CAT AAC AGC AT AGA AAC TTC CTA TCT CTA GG	57	869	Bekele et al. 2010
M. elsdenii	M. elsd-F M. elsd-R	GAC CGA AAC TGC GAT GCT AGA CGC CTC AGC GTC AGT TGT C	58	130	Bekele et al. 2010
S. ruminantium	S. rumi-F S. rumi-R	TGC TAA TAC CGA ATG TTG TCC TGC ACT CAA GAA AGA	53	513	Bekele et al. 2010
C. sticklandii	C. stick-185F C. stick-626-R	ATC AAA GAA TTT CGG ATA GG CAA GTT CAC CAG TTT CAG AG	61	442	This study
C. aminophilum	C.amin-57F C.amin-616R	ACG GAA ATT ACA GAA GGA AG GTT TCC AAA GCA ATT CCA C	57	560	This study

[a] Primers were based on 18S rRNA genes for ciliate protozoa and 16S rRNA genes for bacterial groups and archaea

and the control. Molar proportion of BCVFA was also lower at both the doses of vanillin than the control. Molar proportion of butyrate tended ($P<0.10$) to be greater at the high vanillin dose than the control, but no significant difference was found between the control and the low vanillin dose or between the low and the high vanillin doses.

Table 2 Effect of different doses of vanillin on gas production, degradability of feed substrate, and pH in the in vitro rumen mixed cultures

	Dose of vanillin (mM)			SEM	Contrast	
	0	5	10		Linear	Quadratic
Total gas (ml)	78.9[a]	78.5[a]	74.4[b]	0.59	0.002	0.043
Methane (ml)	18.8[a]	18.4[a]	17.1[b]	0.29	0.005	0.238
DM degradability (%)	73.9[a]	73.5[a]	71.4[b]	0.36	0.003	0.114
NDF degradability (%)	45.2[a]	40.7[a,b]	37.2[b]	1.45	0.008	0.766
pH	6.51[a]	6.48[b]	6.49[b]	0.005	0.018	0.013
Ammonia (mM)	18.8[a]	14.5[b]	10.5[c]	1.13	0.008	0.500

Different superscripts in the same row indicate significant differences ($P\leq0.05$) among the treatments

SEM standard error of mean, DM dry matter, NDF neutral detergent fiber

Effects of vanillin on abundances of methanogens, protozoa, and bacterial species

There were mixed effects of vanillin on the rumen microbial populations in the mixed in vitro rumen cultures (Table 4). Abundances of archaea, *Ruminococcus albus*, *Fibrobacter succinogenes*, *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Streptococcus bovis*, and *C. sticklandii* were not affected by vanillin at 5 or 10 mM, but protozoal population was decreased linearly with increasing vanillin doses. On the other hand, increased abundances were seen with increasing doses of vanillin for *Ruminococcus flavefaciens*, *Prevotella bryantii*, and *Butyrivibrio fibrisolvens* (linearly and quadratically), *Prevotella ruminicola* and *Clostridium aminophilum* (linearly), and *Ruminobacter amylophilus* (quadratically). The sizes of all these bacterial populations were higher at the high vanillin dose than the control.

Effects of essential oils on ammonia concentration, abundances of methanogens, protozoa, and bacterial species

Ammonia concentrations were significantly lower for ORO and CLO than the control, while PEO had little effect on ammonia concentration in the mixed rumen cultures (Table 5). All the microbial groups analyzed were significantly affected by EO, but to different extents depending upon the EO. All EO decreased abundances of protozoa, *S. ruminantium*, *R. amylophilus*, *P. ruminicola*, and *P. bryantii*, with the greatest decrease observed for ORO, followed by CLO and PEO. The populations of *M. elsdenii*, *C. aminophilum*, and *C. sticklandii* were deceased by only ORO, while that of *B. fibrisolvens* was lowered by ORO and CLO, but not PEO. The abundances of the other populations analyzed were not lowered by any of the three EO. In contrast, all EO increased the population of *S. bovis*.

Effects of saponins on ammonia concentration and abundances of methanogens, protozoa, and bacterial species

Saponin decreased ammonia concentration significantly in the mixed rumen cultures (Table 6). Protozoal population was also decreased by saponins. In contrast, the abundances of *S. ruminantium*, *R. amylophilus*, *P. ruminicola*, and *P. bryantii* were increased by saponins, though the magnitude was relatively small (less than one log unit). Saponin had no effect on the growth of *M. elsdenii*, *S. bovis*, *B. fibrisolvens*, *C. aminophilum*, or *C. sticklandii*.

Discussion

The microbiological underpinning of production of ammonia and methane in the rumen and its mitigation has attracted great research interest in the pursuit of a sustainable and environmentally friendly livestock industry. Phytochemicals containing bioactive compounds are of particular interest because of their natural label. Although essential oils and saponins have been evaluated for their effects on protein degradation and ammonia concentration in the rumen (Newbold et al. 2004; Castillejos et al. 2006; Patra 2011a,b), their effect on population dynamics of proteolytic and amino acid-fermenting bacteria has not been examined using molecular tools. The effects of yucca saponins (Wallace et al. 1994) and a blend of EOs (McIntosh et al. 2003) have been evaluated on pure cultures of several rumen bacteria; however, studies using pure culture eliminated the intricate microecological interactions present exist in the complex rumen microbial ecosystem. However, any study did not investigate the effects of the phytochemicals on protein-degrading and amino acid-deaminating rumen bacteria using nucleic acid-based molecular microbial techniques, which is important for understanding the

Table 3 Effect of different doses of vanillin on total volatile fatty acid (VFA) concentration and molar percentages of individual fatty acids in the in vitro rumen mixed cultures

Different letters in the same row indicate significant differences ($P \le 0.05$) among the treatments

SEM standard error of mean, *A/P* acetate to propionate ratio, *BCVFA* branched-chain volatile fatty acids

[a] Effects showed a tendency ($0.05 < P \le 0.10$)

	Dose of vanillin (mM)			SEM	Contrast	
	0	5	10		Linear	Quadratic
Total VFA (mM)	92.8a	94.6a	85.5b	2.15	0.052	0.086
Acetate (%)	57.1	57.7	58.5	0.49	0.099	0.876
Propionate (%)	22.6	22.5	22.3	0.47	0.670	0.685
Butyrate (%)[a]	12.0b	12.3a,b	13.1a	0.26	0.025	0.579
Isobutyrate (%)	1.93	1.66	1.73	0.125	0.301	0.309
Isovalerate (%)	3.51a	2.90b	2.38c	0.147	0.002	0.838
Valerate (%)	2.92a	2.91a	2.12b	0.170	0.016	0.107
A/P	2.53	2.57	2.63	0.075	0.384	0.890
Total BCVFA (%)	5.44a	4.56b	4.11b	0.244	0.002	0.921

Table 4 Effect of different doses of vanillin on abundances (log$_{10}$ copies of *rrs* genes per ml of culture) of archaea, protozoa, and various protein-degrading bacterial species in the in vitro rumen mixed cultures

| | Dose of vanillin (mM) | | | SEM | Contrast | |
	0	5	10		Linear	Quadratic
Archaea	8.23	8.28	8.34	0.053	0.150	0.937
Protozoa	9.57a	9.13b	8.97b	0.121	0.024	0.428
R. albus	7.25	7.25	7.33	0.104	0.620	0.803
R. flavefaciens	8.37b	8.27b	8.94a	0.088	0.004	0.012
F. succinogenes	7.65	7.22	7.24	0.138	0.080	0.138
S. ruminantium	8.22	8.80	8.91	0.333	0.194	0.589
R. amylophilus[a]	8.45a,b	8.65a	8.44b	0.060	0.916	0.031
P. ruminicola	6.60c	6.94b	7.22a	0.079	0.001	0.739
P. bryantii	6.34c	7.36b	8.00a	0.058	<0.001	0.035
M. elsdenii	7.32	7.50	7.61	0.152	0.194	0.589
S. bovis	6.87	6.41	6.73	0.203	0.637	0.166
C. aminophilum	6.34b	6.65b	7.80a	0.152	0.001	0.066
C. sticklandii	6.64	6.96	7.10	0.183	0.234	0.564
B. fibrisolvens	7.21c	7.61b	7.77a	0.035	<0.001	0.028

Different letters in the same row indicate significant differences (*P*≤0.05) among the treatments

SEM standard error of mean

[a] Effects showed a tendency (0.05<*P*≤0.10)

mechanisms of action on degradation of protein and deamination of amino acids in the rumen. We, thus, investigated various phytochemicals (essential oils, vanillin, and saponins) with respect to both ammonia production and population abundances of several protein-degrading and amino acid-deaminating rumen bacteria using real-time PCR systematically. This study shined new light on the complex interactions of some of the commonly investigated phytochemicals and populations of proteolytic and deanimating bacteria.

Vanillin effects on rumen fermentation and abundances of microorganisms

Vanillin is the primary constituent of extract of vanilla beans (*Vanilla planifola*, *Vanilla pompona*, or *Vanilla tahitensis*; Davidson and Naidu 2000). Vanillin has strong antimicrobial activities against a number of bacteria, yeasts, and molds (Davidson and Naidu 2000; Fitzgerald et al. 2004). Although vanillin decreased methane production, it appeared to be less ant-methanogenic compared with other phytochemicals reported earlier, such as origanum, garlic, peppermint, and clove essential oils (Patra and Yu 2012; Patra et al. 2010). Vanillin decreased fiber degradability substantially. Intriguingly, however, vanillin did not reduce populations of the microbial groups analyzed in this study, including methanogens, *R. albus* and *F. succinogenes*, within the 24-h incubation. This suggests that vanillin might be microbiostatic rather than microbicidal. This is consistent with the finding of Fitzgerald et al. (2004) who reported that vanillin at minimum inhibitory concentrations (>15 mM) were bacteriostatic towards *Escherichia coli* and *Lactobacillus* spp. Vanillin decreased total VFA concentrations, which resulted from lowered degradability of the feed substrate. Although major VFA profiles were not influenced by vanillin except butyrate, which tended to increase with increasing doses of vanillin,

Table 5 Effect of essential oils on concentrations of ammonia (mM) and abundances (log$_{10}$ copies of *rrs* genes per ml of culture) of archaea, protozoa, and various protein-degrading bacterial species in the in vitro rumen mixed cultures

	Control	CLO	ORO	PEO	SEM	P-value
Ammonia	28.4a	20.0b	18.6b	23.7a,b	2.02	0.036
Protozoa	8.27a	6.03b	5.60c	5.53c	0.084	<0.001
S. ruminantium	9.37a	8.30c	7.63d	8.82b	0.049	<0.001
R. amylophilus	7.41a	7.08c	7.00c	7.24b	0.044	0.002
P. ruminicola	7.91a	6.92b	5.57c	7.27b	0.143	<0.001
P. bryantii	7.64a	6.66b	5.29c	6.98b	0.136	<0.001
M. elsdenii	6.91a	6.62a	6.05b	6.77a	0.099	0.032
S. bovis	5.14c	6.65b	8.56a	6.53b	0.397	0.009
C. aminophilum	7.99a,b	7.95a,b	6.09c	8.50b	0.212	0.001
C. sticklandii	8.10a	7.80a	6.55b	7.94a	0.111	<0.001
B. fibrisolvens	7.60a	6.53b	6.39b	7.49a	0.059	<0.001

Different superscripts in the same row indicate significant differences (*P*≤0.05) among the treatments

SEM standard error of mean, *CLO* clove oil, *ORO* origanum oil, *PEO* peppermint oil

Table 6 Effect of quillaja saponin on concentrations of ammonia (mM) abundances ($\log_{10}$ copies of *rrs* genes per ml of culture) of archaea, protozoa, and various protein-degrading bacterial species in the in vitro rumen mixed cultures

	Control	Saponin	SEM	*P* value
Ammonia	24.1[a]	18.4[b]	0.62	0.003
Protozoa	9.23[a]	8.51[b]	0.080	0.024
S. ruminantium	9.23[b]	9.55[a]	0.047	0.040
R. amylophilus	8.17[b]	9.46[a]	0.021	0.001
P. ruminicola	7.83[b]	8.07[a]	0.028	0.010
P. bryantii	7.56[b]	7.80[a]	0.053	0.048
M. elsdenii	7.05	6.85	0.187	0.536
S. bovis	6.45	7.12	0.316	0.271
C. aminophilum	7.72	7.93	0.051	0.099
C. sticklandii	7.66	7.81	0.047	0.159
B. fibrisolvens	8.13	7.80	0.114	0.286

Different superscripts in a row indicate significant differences ($P \leq$ 0.05) among the treatments

SEM standard error of mean

molar proportions of minor VFA such as valerate, isovalerate, and branched-chain VFA (BCVFA) were lower in the presence of vanillin. This probably reflects shift in microbial populations and or fermentation pathways in the ruminal cultures. The results of the present study are in agreement with those reported by Castillejos et al. (2006) who observed that vanillin at 0.5 g/L did not change any VFA profile except acetate, but 5 g/L vanillin decreased total VFA and proportions of acetate and BCVFA and increased proportion of propionate in the rumen cultures, though the magnitudes were small. To our knowledge, the present study is the second report on the effect of vanillin on rumen fermentation in the literature.

Ammonia concentrations decreased considerably (by 44 %) when 10 mM vanillin was added to the culture medium, suggesting reduced protein degradation and/or amino acid fermentation. This was further substantiated by the lower proportion of BCVFA and valeric acid, both of which primarily arise from deamination of amino acids. Determination of protein degradation could explore whether vanillin inhibited proteolysis or amino acid fermentation in rumen mixed cultures. Nonetheless, to understand these effects of vanillin, we quantified the abundances of major known protein-degrading rumen bacteria, i.e., *P. ruminicola*, *R. amylophilus*, *B. fibrisolvens*, *S. ruminantium*, *P. bryantii*, and *S. bovis* (Wallace et al. 1997), and amino acid-fermenting bacteria, i.e., *C. aminophilum*, *C. sticklandii*, *M. elsdenii*, *P. ruminicola*, *B. fibrisolvens*, *S. ruminantium*, and *S. bovis* (Wallace et al. 1997; Russell et al. 1988) using real-time PCR. Surprisingly, none of the above proteolytic or amino acid-fermenting bacteria were lowered in abundance, and the abundance of *P.*

ruminicola, *P. bryantii*, *C. aminophilum*, and *B. fibrisolvens* were increased by vanillin despite significant reduction in ammonia production. Physiological and ecological studies employing (meta)transcriptomic and (meta)proteomic analysis on these and other rumen bacteria are needed to determine if vanillin directly inhibits proteolytic and amino acid-fermenting bacteria in the rumen.

Protozoa contribute significantly to protein degradation in the rumen, and defaunation often results in reduced ammonia concentration in the rumen (Wallace and McPherson 1987; Firkins et al. 2007). Thus, the reduced protozoa population might be partially responsible for lowered ammonia production by vanillin. However, the ammonia reduction magnitude observed in this study exceeded what is usually observed from defaunation alone. Tannin can interact with protein forming tannin–protein complexes that are recalcitrant to microbial degradation in the rumen (Patra and Saxena 2011). Vanillin has been shown to interact with protein such as albumin and casein in a phosphate buffer (Chobpattana et al. 2002), but it remains to be determined if vanillin can also form recalcitrant vanillin–protein complex in the rumen.

Effects of essential oils on protein-degrading microorganisms

Essential oils can exhibit broad antimicrobial activities, and their potency differs among EO components and species of bacteria in the rumen (McIntosh et al. 2003; Patra 2011a,b; Patra and Yu 2012). The ability of a few EOs to decrease ammoniagenesis has also been noted in other studies (Cardozo et al. 2005; Castillejos et al. 2006). Consistent with these previous studies, both CLO and ORO substantially lowered ammonia concentrations in the mixed in vitro rumen cultures, while PEO numerically reduced ammonia concentration (Table 5). The reduced ammonia production mirrors the reduced abundances of the major protein-degrading and amino acid-fermenting bacteria except *S. bovis* (Table 5). Interestingly, the abundances of *S. bovis* increased in response to all the EOs tested, probably due to reduced competition from other bacteria that were inhibited by the EOs. It is also evident that different bacteria declined to different extent in response to the same EO, a finding corroborating the finding of McIntosh et al. (2003), who showed that pure cultures of *C. sticklandii* and *P. anaerobius* were more susceptible than *P. ruminicola* and *P. bryantii*, while *S. bovis* was the least susceptible to EO. Evans and Martin (2000) also noted that thymol was more inhibitory to *S. ruminantium* than to *S. bovis*.

In a previous study (Patra and Yu 2012), methane production and ammonia concentrations were lowered by EO, but these responses were accompanied by reduced fiber digestibility and concentrations of total VFA. Thus, to achieve

substantial decreases in methane production and ammonia concentrations, nutrient supply to animals may be compromised. For instance, in a study with EO, digestibilities of nutrients have been found to decrease in cattle fed daily 1.0 g of an EO mixture (Beauchemin and McGinn 2006) and 1.6 g of cinnamaldehyde (Yang et al. 2010). There is also a report that EO compounds from oregano leaves lowered methane production considerably without much influence on nutrient digestibility (Hristov et al. 2013).

The three EOs exhibited different antimicrobial activities, probably due to the presence of different active components. ORO was the most antimicrobial followed by CLO and PEO. CLO contains eugenol (a phenylpropanoid) as its main active component, while ORO has thymol (a monoterpinoid monoclyclic phenol) and PEO has menthol (a monoterpinoid monoclyclic non-phenol) as their active components. Several mechanisms have been proposed for their antimicrobial properties, with chemical structures and physical properties being the major determinants of their antimicrobial potency (Dorman and Deans 2000). The hydrophobic property of EO permits them to partition into the lipid cell membranes, disturbing the cell membrane integrity and stability and leading to leakage of cell contents (Burt 2004). The hydroxyl group and their relative position in the phenolic structures (in the case of thymol and eugenol) were thought to be important attributes that influence the antimicrobial effects of EO (Dorman and Deans 2000; Ultee et al. 2002). This may explain the greater inhibition of ammonia production by ORO than by PEO and CLO.

Effects of saponins on protein-degrading microorganisms

Although saponins reduced the ammonia concentrations by 20 %, it did not reduce the abundance of the major protein-degrading or ammoniagenic bacteria analyzed (Table 6). As did vanillin, saponins suppressed the growth of protozoa, consistent with the antiprotozoal properties of saponins reported in other studies (Patra and Saxena 2009b; Patra et al. 2012). Degradation of feed protein and predation of bacteria by protozoa play a major role in protein degradation in the rumen (Wallace and McPherson 1987). In this study, reduction of protozoal activity probably largely contributed to the reduced ammonia concentrations, but it is also possible that saponins elicited a direct antimicrobial effect on proteolytic or deaminating species of ruminal bacteria that have not yet been isolated in pure culture. A few studies have reported conflicting effects of yucca saponin on the growth of several pure cultures of rumen bacteria. These include inhibition of *S. ruminantium*, *M. elsdenii*, and *S. bovis* (Katsunuma et al. 2000); inhibition of *S. bovis* and *B. fibrisolvens*, no effect on *S. ruminantium*, but stimulation to *P. ruminicola* (Wallace et al. 1994); and inhibition of *S. bovis*, *P. bryantii*, and *R. amylophilus*, but stimulation to *S.*

ruminantium (Wang et al. 2000). No study has been reported that examined the effect of quillaja saponin, which contains different sapogenin than yucca saponin, on individual pure as well as mixed cultures of protein-degrading rumen bacteria. In the present study, quillaja saponin was found to stimulate some of the protein-degrading bacteria analyzed while not affecting others in the mixed rumen cultures. More studies using pure cultures of rumen bacteria are needed to determine the effect of quillaja saponin on the growth and metabolism of rumen bacteria.

In summary, vanillin can lower methane and ammonia production by rumen microbiome in vitro, but these effects are probably not associated with direct inhibition to archaea, proteolytic or ammoniagenic bacteria. Similarly, quillaja saponin reduced ammonia production by rumen microbiome in vitro but did not decrease the populations of major known proteolytic or ammoniagenic bacteria. The antiprotozoal activity of saponins and vanillin may partly explain the reduced ammonia production. Protein–vanillin complex formation may hinder protein degradability by rumen microorganisms, subsequently decreasing amino acid deamination. Vanillin and saponin may also exert an antimicrobial effect on uncultured species of protein-degrading ruminal bacteria. However, EO lowered ammonia concentrations, probably due to direct inhibition to proteolytic and ammoniagenic rumen bacteria. Collectively, the phytochemicals tested in this study have potential to mitigate methane emission and ammonia excretion from ruminant animals, but future studies including (meta)transcriptomic and (meta)proteomic analysis of the ruminal microbiome are needed to better understand their modes of action.

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