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A meta-analysis of effects of chemical composition of incubated diet and bioactive compounds on *in vitro* ruminal fermentation☆

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ABSTRACT

This study examined the role of supplementation of several bioactive compounds (BC) and the chemical composition of the diet used as substrate for *in vitro* incubations, on *in vitro* ruminal fermentation profile and nutrient degradation. A meta-analytical approach was used to weigh the sample size used in each experiment, and account for the random effect of each as well as unequal variance among studies. A total of 20 recently conducted experiments with 354 treatments, each including one control (*i.e.*, no BC supplementation), fulfilled the criteria for inclusion. Doses of BC supplementation varied from 0.03 to 500 mg/g dry matter (DM) of incubated diet. Contents of crude protein (CP) and neutral detergent fibre (NDF) of the incubated diets (DM basis) ranged from 139 to 189 g/kg and 160 to 420 g/kg, respectively. Results indicate that supplementation of BC linearly decreased (137.4 *versus* 116.5 mmol/L; P<0.05) concentration of total volatile fatty acids (VFA) and proportion of acetate (P<0.05). Also, the concentration of ammonia in the *in vitro* rumen fluid was lower with BC supplementation (22.9 *versus* 15.6 mg/dL; P<0.05). Analysis by backward elimination correlation analysis revealed that inclusion of the chemical composition of the incubated diet into the model with BC supplementation improved the accuracy of estimation of responses of fermentation variables. Thus, higher NDF and CP contents of the substrate and higher BC dosage were associated with lower concentrations of total VFA (r^2 = 0.54), whereas both lower CP contents of the substrate and BC supplementation lowered the concentration of ammonia (r^2 = 0.32). This analysis showed negative associations between BC supplementation and *in vitro* disappearance of DM and NDF, and positive correlations with dietary NDF content. In contrast, higher BC inclusion and lowering NDF content in the diet was accompanied with decreased *in vitro* CH_4 formation (r^2 = 0.21). Results indicate that BC supplementation and chemical composition of the incubated diet are determining factors which impact responses of *in vitro* ruminal fermentation and degradation.

Abbreviations: aNDFom, neutral detergent fibre measured using heat stable amylase and exclusive of residual ash; BC, bioactive compounds; CI, confidence intervals; CP, crude protein; DM, dry matter; IVDMD, *in vitro* DM disappearance; IVNDFD, *in vitro* NDF disappearance; NDF, neutral detergent fibre; RMSE, root mean square error; VIF, variance inflation factor; VFA, volatile fatty acids.

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1. Introduction

Improving the efficiency of energy and protein utilization is a major goal in ruminant nutrition because energy and ammonia losses impact animal production and the environment. However this goal can be achieved by shifting the volatile fatty acid (VFA) profile towards propionate, without reducing total VFA production, while reducing protein degradation and CH_4 yields in the rumen. In this context, ruminant nutritionists have dedicated their research to identify effective compounds and dietary formulations to modify ruminal fermentation. As ruminal fermentation is due to microbes (*i.e.*, bacteria, archaea, fungi, ciliate protozoa), substances affecting activity of microbes, such as antibiotic ionophores (McGuffey et al., 2001), could modify ruminal fermentation. However, because the use of antibiotics has been banned in the European Union since 2006, there has been interest in exploiting natural feed additives, such as naturally occurring bioactive compounds, as modifiers of ruminal fermentation.

Bioactive compounds (BC) are defined as essential and non-essential compounds which occur in the nature and are a part of the feed-food chain (Biesalski et al., 2009). Despite their often very low quantities, BC possess several health-promoting properties (Biesalski et al., 2009), often antimicrobial activity, with some of them used as traditional medicines (Reichling, 2009). Since 2004 there have been a number of *in vitro* studies investigating effects of various BC, including essential oils, on ruminal microbial fermentation and CH_4 mitigation (*e.g.*, Cardozo et al., 2004; Castillejos et al., 2006; Benchaar et al., 2007). However, the effectiveness of BC on modifying ruminal fermentation has been inconsistent and inconclusive. The discrepancy of results could be attributed to different chemical structures and types as well as the different doses of BC used (Busquet et al., 2006; Benchaar et al., 2007). In addition, it appears that effects of some metabolites in BC could depend on ruminal pH (Cardozo et al., 2005) and type of diet used for *in vitro* incubation (Calsamiglia et al., 2007).

It is clear that there are several factors and, potentially, interactions among factors that modify the expected responses of BC relative to ruminal fermentation. This leads to conflicting results among studies, while evaluation of multiple relationships can generally not be accomplished in a single study. A meta-review of the literature may be used to resolve such discrepancies (St-Pierre, 2001). Results of a meta-analysis of BC *in vitro* may explain responses *in vivo*.

Our objective was to examine effects of BC on *in vitro* ruminal fermentation profiles and nutrient degradation using a meta-analysis approach. We focused particularly on effects associated with the dose of BC supplemented and the chemical composition of the incubated diets. Measurements of interest included the contents of neutral detergent fibre (NDF) and crude protein (CP) in the incubated diet as well as *in vitro* disappearance of dry matter (DM) and NDF, concentrations and proportions of the major VFA (*i.e.*, acetate, propionate, butyrate), ammonia, and CH_4 formation.

2. Materials and methods

2.1. Data search

A literature search was conducted using public data search generators, such as Pubmed, Google scholar, Sciencedirect, and Scopus, as well as contact with researchers in the field to identify published articles on effects of BC on rumen fermentation characteristics *in vitro*. The search strategy aimed to identify articles which contained specific data on experiments examining effects of supplementing different types of BC on *in vitro* rumen fermentation characteristics, particularly on total VFA concentrations and proportions of major VFA, *in vitro* disappearance of DM (IVDMD) and NDF (IVNDFD) as well as ammonia concentrations. The following key words, in different combinations, were used for our search: BC, essential oils, *in vitro* rumen fermentation, IVDMD and IVNDFD. The most commonly reported parameters were identified to be the concentration of total VFA and their major components (*i.e.*, acetate, propionate, butyrate) as well as ammonia concentration, whereas IVDMD and IVNDFD were reported in fewer studies. Details on chemical composition such as NDF – including both determination of NDF without and with heat stable amylase (aNDF), inclusive and exclusive (NDFom) of residual ash – and CP contents of the incubated diets were also extracted from the articles. A database was built using these data in an Excel spreadsheet. A list of publications reviewed for the study with their respective BC and measured response variables is in Table 1. Studies included in this meta-analysis included experiments published in several peer-reviewed Journals from 2004 to 2011. As shown in Table 1, there were 70 BC compounds tested from different studies that were included.

2.2. Inclusion criteria for the study

Experiments were included or excluded based on the following quality assessment criteria: sufficient data on BC and their supplementation dose, chemical composition of the incubated diets, fermentation parameters, clear experimental design with sufficient replicates, and randomization of treatment groups, statistical analysis and intra-study error. Supplementation dose of BC was provided as mg/L culture or mg/g DM of incubated diets; when only the first dose was provided this was converted into mg/g DM based on the volume of the incubation culture and the amount of incubated substrate. Particular emphasis was also placed on the presence of a control treatment (*i.e.*, only substrate without BC) in every experiment. To contrast effects of BC supplementation, besides the control treatment, two diverse groups with BC supplementation were considered. The first group involved doses of BC from >0 to 100 mg/g DM substrate (*i.e.*, low group), whereas treatments

Table 1
List of references with their bioactive compounds (BC) tested and the respective experimental variables[a] included in the meta-analysis.

	Types of BC[b]	VFA	NH_3	IVDMD	IVNDFD
Benchaar et al. (2007)	13, 16, 18, 24, 49, 64, 67	*	*	*	*
Busquet et al. (2005a)	1, 3, 21, 27	*	*	*	*
Busquet et al. (2005b)	16, 27	*	*	*	*
Busquet et al. (2005c)	13, 16, 24	*	*		
Busquet et al. (2006)	4, 5, 8, 11, 13, 14, 16, 18, 22, 24, 25, 27, 29, 49, 66	*	*		
Cardozo et al. (2004)	4, 10, 13, 16, 20, 27, 60	*	*		
Cardozo et al. (2005)	4, 10, 13, 16, 24, 27, 60	*	*		
Castillejos et al. (2005)	20	*	*	*	*
Castillejos et al. (2006)	67, 24, 30, 42, 67, 69	*	*		
Castillejos et al. (2007)	20	*	*	*	
Castillejos et al. (2008)	18, 34, 37, 49, 57, 58, 61, 66, 67	*	*		
Chaves et al. (2008)	4, 16, 27, 36, 50	*	*		
Fraser et al. (2007)	16	*	*	*	*
Hristov et al. (2008)	6, 7, 9, 12, 15-18, 23, 26, 28, 31, 32, 35–38, 41, 44–47, 49, 51–53, 55–59, 62-67, 70	*	*		
Kongmun et al. (2010)	19, 27	*	*		
Kung et al. (2008)	20	*	*		
Lourenço et al. (2008)	16, 24, 54, 68	*			
Mohammed et al. (2004)	33	*	*	*	*
Soliva et al. (2011)	2, 27, 39, 43	*	*	*	*
Spanghero et al. (2008)	16, 48, 49, 67	*	*		

[a] VFA = volatile fatty acids; NH_3 = ammonia; IVDMD = *in vitro* dry matter disappearance; IVNDFD = *in vitro* neutral detergent fibre disappearance.
[b] Types of bioactive compounds tested were: (1) allicin; (2) allyl isothiocyanate; (3) allyl mercaptan; (4) anethole; (5) anise oil; (6) basil oil; (7) bergamot oil; (8) cade oil; (9) camphor oil; (10) capsaicin; (11) capsicum oil; (12) caraway oil; (13) carvacrol; (14) carvone oil; (15) cedar wood oil; (16) cinnamaldehyde + cinnamon oil; (17) citronella; (18) clover oil; (19) coconut oil; (20) CRINA (trade mark of essential oils mixture); (21) diallyl sulphide; (22) dill weed oil; (23) eucalyptus oil; (24) eugenol; (25) fenugreek oil; (26) frank myrrh oil; (27) garlic oil; (28) gardenia oil; (29) ginger oil; (30) guaiacol; (31) hibiscus; (32) honeysuckle; (33) horseradish oil; (34) hyssop oil; (35) jasmine oil; (36) juniper oil; (37) lavandin oil; (38) lemongrass; (39) levulinic acid; (40) lilac oil; (41) lily oil; (42) limonene; (43) lovastatin; (44) magnolia; (45) musk; (46) neroil; (47) nutmeg; (48) orange peel; (49) oregano oil; (50) P-cymene; (51) patchouli; (52) peppermint; (53) petitgrain oil; (54) quercetin; (55) rose gerin; (56) provence rose oil; (57) rosemary oil; (58) sage oil; (59) sandalwood; (60) sarsaponin; (61) savory oil; (62) siberian pine; (63) spearmint; (64) sweet orange; (65) tangerine; (66) tea tree oil; (67) thymol; (68) triterpene oil; (69) vanillin; (70) wintergreen oil.

including >100 mg BC/g DM were considered as high doses (*i.e.*, high group). Treatments with compounds which were not classified as BC compounds, including antibiotic ionophores, were not included in the database.

2.3. Data extraction and description of database

A total of 20 studies (354 treatments including one control – substrate without BC inclusion – in every experiment) met the eligibility criteria for meta-analysis. The recorded data included authors, journal/year of publication, experiment design, type of incubated substrate, as well as the contents of CP and NDF in the diet. Other information extracted from relevant articles were concentration of VFA in the incubation liquid, proportions of major VFA (*i.e.*, acetate, propionate, butyrate), concentration of ammonia, IVNDFD and IVDMD, and the respective standard error of each variable.

Calculation of the CH_4 formed during *in vitro* fermentation was according to Moss et al. (2000) as:

$$CH_4 \text{mmol}/100\text{molVFA} = 0.45(\text{acetate}) - 0.275(\text{propionate}) + 0.4(\text{butyrate}).$$

A summary of the response variables considered in this meta-analysis with a simple statistical description is in Table 2. Ranges in the doses of BC were from very low (0.03 mg/g DM) to extremely high (500 mg/g DM) of the incubated diet. The content of CP and the amount of NDF of the incubated diets ranged from 139 to 189 g/kg and 160 to 420 g/kg, respectively. Also, the range of the response variables measured differed considerably. Particularly, concentrations of VFA and ammonia indicated strong variations among studies included (Table 2).

2.4. Data analysis

To evaluate the *in vitro* fermentation responses to predictor variables, all data were subjected to mixed modelling analysis using PROC MIXED (SAS, 2011), and considering the random effect of the study (St-Pierre, 2001), as:

$$Y_{ij} = \alpha_0 + \beta_1 X_{ij} + s_i + b_i X_{ij} + e_{ij}$$

where: Y_{ij} = the expected outcome for the dependent variable Y observed at level j ($j = 2, \ldots, n$) of the predictor variable X in the study i, while n is the number of treatment means in study i, α_0 = the overall intercept across all studies (fixed effect), β_1 = the overall regression coefficient of Y on X across all studies (fixed effect), X_{ij} = the value j of continuous variable X in

Table 2
Statistical description of doses of bioactive compounds (BC), chemical composition of incubated diets, and *in vitro* rumen fermentation response variables included in the database.

	n_{Treat} [a]	n_{Ref} [b]	Mean	SE	Min	Max	Median
Doses of BC (mg/g dry matter)[c]	354	20	45.6	5.85	0.03	500	45.0
Chemical composition of diets (g/kg dry matter)							
Neutral detergent fibre	289	18	280	3.60	160	429	280
Crude protein	294	19	168	0.60	139	189	173
Response variables[d]							
Total VFA (mmol/L)	354	20	124	2.63	11.2	279	123
Methane (mmol/100 mol VFA)	354	20	23.5	0.28	9.80	36.8	23.8
Acetate (mol/100 mol VFA)	354	20	55.4	0.53	53.1	69.9	57.7
Propionate (mol/100 mol VFA)	354	20	21.8	0.34	17.8	37.0	21.3
Butyrate (mol/100 mol VFA)	354	20	11.5	0.22	8.89	33.1	10.7
Acetate:propionate	354	20	2.66	0.04	2.01	4.71	2.63
Ammonia (mg/dL)	344	19	19.8	0.72	6.70	102	21.4
IVDMD	49	8	0.52	0.019	0.25	0.72	0.57
IVNDFD	43	7	0.30	0.014	0.12	0.42	0.34

[a] Number of treatments means including the controls (no inclusion of essential oils).
[b] Number of experiments included in this study.
[c] The controls are excluded.
[d] VFA = total volatile fatty acids; IVDMD = *in vitro* DM disappearance; IVNDFD = *in vitro* neutral detergent fibre disappearance; CH_4 was estimated according to the equation 0.45 (acetate) − 0.275 (propionate) + 0.4 (butyrate) by Moss et al. (2000).

study i, s_i = the random effect of the study i ($i = 1, \ldots, 20$), b_i = the random effect of study i on the regression coefficient of Y on X in study i, and e_{ij} = the unexplained error. Thus, the random effect components of the model include $s_i + b_i X_{ij} + e_{ij}$, and the distributions were: $e_{ij} \sim iid\, N(0, \sigma_e^2)$ and $\begin{bmatrix} S_i \\ b_i \end{bmatrix} \sim iid\, N\left[\left(\frac{0}{0}\right), \Sigma\right]$, which assumes that e_{ij} is normally distributed with a mean of 0 and constant variance, and that s_i and b_i are normally distributed, have means of 0 and Σ is their variance-covariance matrix:

$$\Sigma = \begin{bmatrix} \sigma_s^2 & \sigma_{sb} \\ \sigma_{sb} & \sigma_b^2 \end{bmatrix}$$

An unstructured variance–covariance structure matrix (TYPE = UN) was adopted to avoid the positive correlation between the intercepts and slopes, as suggested by St-Pierre (2001). To take the unequal variance among studies into consideration, the dependent variable was weighted by the reciprocal of its squared SE.

To evaluate effects of chemical composition as well as interactions among diverse dietary factors, several predictor variables were also tested using the backward elimination technique of SAS (2011), similarly to the algorithm reported by Zebeli et al. (2008). In this case, to limit model overparameterization, a variance inflation factor (VIF) less than 10 (which assumes no significant multicollinearity among predictor variables tested) for every continuous independent variable tested was assumed (Neter et al., 1996).

3. Results

3.1. Effects of BC supplementation

Associations between BC supplementation and *in vitro* fermentation parameters are in Table 3. Increasing the level of BC supplementation linearly decreased total VFA concentrations ($P<0.05$) and affected the proportions of individual VFA. Thus, acetate was lower with BC supplementation compared to control ($P<0.05$). No effects of BC dosage occurred on butyrate formation and the acetate:propionate ratio. Ammonia concentrations decreased with BC supplementation ($P<0.05$). In particular, high dosages of BC (>100 mg BC/g DM) decreased ammonia concentration by about 32% compared to control treatment (*i.e.*, no BC dosing).

Data indicate that supplementation of BC (>0–100 mg/g DM) numerically decreased IVDMD and IVDMD but differences did not reach significance due to highly variable results among studies, especially when dosages above 100 mg BC/g DM were used. *In vitro* CH_4 formation was not inhibited by BC supplementation.

3.2. Effects of chemical composition of the incubated diet and BC supplementation

The backward elimination analysis (Table 4) showed that including the chemical composition of the incubated diet in the same model with BC supplementation increased the accuracy of prediction of several variables, as compared with when only individual factors were tested separately (Table 3). Accordingly, total VFA concentration was not only affected by BC supplementation but also by the diet composition (RMSE = 20.87; $r^2 = 0.54$). The analysis indicated that increasing

Table 3
Variables of *in vitro* rumen fermentation[c] in response to the supplementation of different doses of bioactive compounds (BC) as tested by mixed modelling.

	Supplementation doses of BC[b]			P[d]	
	None	Low	High	NO *vs.* BC	Lin
VFA (mmol/L)	137.4 ± 5.67[a]	124.7 ± 3.07[b]	116.5 ± 8.32[b]	0.019	0.041
mol/100 mol VFA					
Acetate	57.7 ± 1.18[a]	54.5 ± 0.64[b]	54.4 ± 1.74[a,b]	0.032	0.096
Propionate	22.9 ± 0.78[a]	21.2 ± 0.42[b]	23.0 ± 1.14[a,b]	0.386	0.977
Butyrate	12.0 ± 0.51	11.7 ± 0.27	11.9 ± 0.75	0.785	0.922
Acetate:propionate	3.03 ± 0.19	2.76 ± 0.10	2.54 ± 0.27	0.117	0.157
Ammonia (mg/dL)	22.9 ± 1.16[a]	21.1 ± 0.64[b]	15.6 ± 1.46[c]	0.002	0.001
IVDMD	0.57 ± 0.022	0.53 ± 0.020	0.55 ± 0.104	0.588	0.914
IVNDFD	0.33 ± 0.031	0.25 ± 0.021	0.35 ± 0.100	0.681	0.920
Methane (mmol/100 mol VFA)	24.4 ± 0.63	23.4 ± 0.34	22.9 ± 0.93	0.109	0.168

[a] A meta-analysis approach was used to account for inter- and intra-experimental variation and random effect of the study (data are shown as least-square means ± standard error of the mean).
[b] NONE = no supplementation of BC (0 mg BC/g DM substrate); LOW = supplementation of >0–100 mg BC/g dry matter substrate; HIGH = supplementation of >100 mg BC/g DM.
[c] VFA = volatile fatty acids; IVNDFD = *in vitro* disappearance of neutral detergent fibre, IVDMD = *in vitro* disappearance of DM.
[d] P indicating contrasts between no supplementation and supplementation of BC (NONE *vs.* BC), as well as the linear contrast of BC supplementation; within a row, means bearing different letters differ at P<0.05.

NDF content of the diet decreased total VFA production (P<0.001). Additional to BC, the dietary composition also affected individual VFA proportions. Thus, increasing the NDF content of the incubated diet increased the proportions of acetate (P<0.05; RMSE = 4.95; r^2 = 0.15) at the expense of propionate (P<0.001; RMSE = 4.47; r^2 = 0.19), which was also reflected in an increased acetate:propionate ratio (P<0.001; RMSE = 0.61; r^2 = 0.20). Butyrate formation was mainly influenced by the chemical composition of the diet (RMSE = 1.20; r^2 = 0.78). Thus, with increasing amount of CP, butyrate formation decreased linearly (P<0.001), whereas it increased with higher dietary NDF content (P<0.001). Dietary CP content promoted *in vitro* ammonia formation (RMSE = 4.43; r^2 = 0.32), whereas it was linearly depressed by BC supplementation (P<0.05).

The backward elimination analysis confirmed that, when NDF and CP content of the diet were included in the model, both IVDMD and IVNDFD were affected by BC addition. With increasing BC supplementation, IVDMD and IVNDFD linearly decreased (P<0.001). Furthermore, dietary NDF and CP concentrations affected *in vitro* nutrient disappearance. Hence with increasing CP content, both IVDMD and IVNDFD linearly decreased (P<0.001), whereas both increased with higher NDF concentration (IVDMD, P<0.05; and IVNDFD, P<0.001).

In contrast to the mixed model procedure, backward elimination analysis revealed that, when NDF content of the diet was included in the analysis, a linear influence of BC on CH_4 formation occurred (RMSE = 3.81; r^2 = 0.21). With increasing BC dosage, CH_4 formation was slightly inhibited (slope = −0.016; P<0.05), whereas increasing levels of NDF in the diet linearly promoted CH_4 formation (P<0.001).

4. Discussion

This study summarizes information from 20 studies published during the last 7 years to examine the role that supplementation dose of BC and the chemical composition of the substrate play on *in vitro* ruminal fermentation. A meta-analysis approach was used to weigh the sample size used in each experiment, and to account for the random effect of each experiment and the unequal variance among studies, due to their experimental differences. By screening a broad range in the supplementation dosage of BC, the study revealed dose–response relationships for several *in vitro* rumen fermentation variables. Results imply that doses of BC above 100 mg/g DM do not necessarily modify *in vitro* ruminal fermentation in a dose–response manner. This may have implications for *in vivo* conditions, because supplementation of high doses of BC reported *in vitro* may not have physiological relevance (Calsamiglia et al., 2007). Hence, only low to moderate BC doses are feasible *in vivo*. Another outcome of our analysis was that, besides the dose of BC, the diet composition (*i.e.*, NDF and CP content), largely modified the effect of BC supplementation on fermentation variables.

Bioactive compounds including essential oils are complex mixtures of secondary metabolites of plants with highly variable chemical composition (Biesalski et al., 2009; Benchaar and Greathead, 2011). Therefore, the different BC likely affected microbial growth and activity differently, as reflected in *in vitro* fermentation patterns. The outcome of this meta-analysis confirms observations from individual studies (*e.g.*, Oh et al., 1967; Cardozo et al., 2004; Castillejos et al., 2006; Benchaar et al., 2007) supporting a general inhibiting effect of BC on microbial activity, as indicated by the linear decrease in the VFA and ammonia concentration. Generally, it is assumed that gram-positive bacteria are more inhibited by BC than gram-negative due to their simple cell membrane compared to the more complex cell wall of gram-negative bacteria (Cox et al., 2001; Cimanga et al., 2002). Thus, shifts in bacterial composition may overall reduce bacterial fermentative activity and selectively favour less BC-sensible gram-negative bacteria. Some studies observed a decrease in protozoal numbers when BC were used (Agarwal et al., 2009; Soliva et al., 2011), which may explain changes in fermentation patterns. About 25% of the ruminal methanogens are associated with protozoa and the latter were account for 10–25% of ruminal CH_4 production

Table 4
Best fit equations showing the response of different *in vitro* variables to changes in the supplementation of bioactive compounds (BC) and the chemical composition of the substrate using backward elimination technique.

variable (*Y*)	Dietary factor[a]	Parameter estimates				Model statistics[b]			
	(*X*)	Intercept	$SE_{Intercept}$	Slope	SE_{Slope}	RMSE	r^2	VIF	P
Total VFA (mmol/L)		270.5	19.40			20.87	0.54		
	CP, g/kg DM			−0.1988	0.1164			1.07	0.088
	NDF, g/kg DM			−0.3563	0.0204			1.08	<0.001
	BC, mg/g DM			−0.077	0.0425			6.46	0.071
	BC^2, mg/g DM			0.0003	0.0001			6.66	0.008
Acetate (mol/100 mol VFA)		54.19	1.36			4.95	0.15		
	NDF, g/kg DM			0.0159	0.0047			1.10	0.009
	BC, mg/g DM			−0.031	0.010			6.35	0.023
	BC^2, mg/g DM			0.00008	0.00002			6.42	0.020
Propionate (mol/100 mol VFA)		40.17	4.15			4.47	0.19		
	CP, g/kg DM			−0.0442	0.0249			1.07	0.077
	NDF, g/kg DM			−0.0338	0.0043			1.08	<0.001
	BC, mg/g DM			0.017	0.009			6.46	0.054
	BC^2, mg/g DM			−0.00005	0.00002			6.66	0.052
Butyrate (mol/100 mol VFA)		7.14	1.11			1.20	0.78		
	CP, g/kg DM			−0.0350	0.0066			1.06	<0.001
	NDF, g/kg DM			0.0374	0.0011			1.04	<0.001
	BC^2, mg/g DM			0.0005	0.0001			1.03	0.004
Acetate:propionate		0.066	0.005			0.61	0.20		
	CP, g/kg DM			0.0079	0.0005			1.07	0.019
	NDF, g/kg DM			0.0046	0.0005			1.08	<0.001
	BC, mg/g DM			−0.0026	0.0012			6.46	0.032
	BC^2, mg/g DM			0.00006	0.00003			6.66	0.037
Ammonia (mg/dL)		44.09	2.36			4.43	0.32		
	CP^2, g/kg DM			0.2360	0.0236			1.04	<0.001
	BC, mg/g DM			−0.077	0.008			6.25	0.002
	BC^2, mg/g DM			0.00004	0.00002			6.28	0.061
IVDMD coefficient		1.063	0.0513			5.51	0.59		
	CP, g/kg DM			−0.0032	0.00031			1.07	<0.001
	NDF, g/kg DM			0.00016	0.00005			1.08	0.004
	BC, mg/g DM			−0.0014	0.00011			6.04	<0.001
	BC^2, mg/g DM			0.000005	0.0000002			6.06	<0.001
IVNDFD coefficient		1.282	0.0226			2.24	0.64		
	CP, g/kg DM			−0.0061	0.00227			1.07	<0.001
	NDF, g/kg DM			0.00025	0.00002			1.08	<0.001
	BC, mg/g DM			−0.0017	0.00004			6.46	<0.001
	BC^2, mg/g DM			0.000006	0.0000001			6.66	<0.001
CH_4 (mmol/100 mol VFA)		15.87	1.04			3.81	0.21		
	NDF, g/kg DM			0.0320	0.0036			1.04	<0.001
	BC, mg/g DM			−0.016	0.077			9.35	0.033
	BC^2, mg/g DM			0.00004	0.00001			6.42	0.024

[2] indicates square term of the predictor variables (dietary factors).

[a] CP = crude protein; BC = essential oils supplementation dose, IVNDFD = *in vitro* disappearance of neutral detergent fibre, IVDMD = *in vitro* disappearance of DM.

[b] RMSE = root mean square error; r^2 = coefficient of determination, VIF = variance inflation factor (VIF < 10 is assumed to show no significant multicollinearity among predictor variables).

(Newbold et al., 1995). Thus, it cannot be completely excluded that anti-protozoal activity of BC might partly be responsible for the inhibited CH_4 formation found in several studies (Soliva et al., 2011).

Although data of this meta-analysis come from *in vitro* experiments, and particular caution should be paid when extrapolating *in vitro* data to *in vivo* conditions, the reduction in the VFA concentrations in response to BC supplementation may have negative effects on production efficiency of ruminants. This assumption is based on the reality that VFA are the main energy substrates of ruminants (Chaves et al., 2008; Morgavi et al., 2010), and any decrease in ruminal VFA formation would subsequently reduce the energy supply to the host (Aschenbach et al., 2011). However a less rapid release of VFA in rumen fluid may be desirable from the rumen health point of view, because this event can lower the risk of rumen acidosis (Zebeli et al., 2012).

Although BC supplementation was not associated with marked depressions of IVDMD and IVNDFD, the present meta-analysis and in particular the backward regression analysis, supports the overall view that BC supplementation generally impairs nutrient degradation (Calsamiglia et al., 2007; Hart et al., 2008), in particular when dietary contents of CP decreased and NDF was higher. Reduction of proteolysis, peptidolysis, and deamination by increasing doses of BC (Calsamiglia et al., 2007), in turn, may be advantageous for the host animal to increase the amount of dietary protein reaching the small intestine. Indeed, there are indications that effects of BC on proteolysis might mainly lie in the final step, deamination of amino acids (McIntosh et al., 2003), and this would increase the supply of dietary amino acids to the duodenum. Accordingly,

our results showed that high doses of BC can reduce ammonia concentration by more than 30%. This might be due to a higher sensitivity of "hyper ammonia producing (HAP) bacteria" towards some BC (McIntosh et al., 2003). In contrast, *in vivo*, reduced proteolysis in the rumen may lower ammonia availability and negatively affect microbial protein synthesis, and this in turn may impair fibre degradation, feed intake, and milk production (Nocek and Russell, 1988). Focusing on our results, only high BC dosages strongly decreased the ammonia concentration, indicating that high levels of BC might not necessarily be needed if the availability of ammonia might be a limiting factor *in vivo*. As shown by the results of the backward analysis, ammonia concentration, and hence the degree of proteolysis, depends also on the type of the diet (*i.e.*, amount of CP in the diet), indicating that the dose of BC can be optimised based on the content of CP of the diet. Controlled proteolysis is often desired to better synchronize N and energy release in the rumen (Nocek and Russell, 1988; Russell et al., 1992). Thus, depending on diet composition and feeding strategy, reduced proteolysis in the rumen through BC supplementation might be helpful to lower excessive ammonia levels in the rumen, and, therefore, lead to lower ammonia losses.

Besides the total VFA concentration, proportions of individual VFA were modified by BC supplementation. For example, acetate proportion decreased with low doses of BC in some studies (*e.g.*, Cardozo et al., 2005), which is seen as beneficial in terms of reduction in ruminal CH_4 generation (Calsamiglia et al., 2007). Despite the reduced acetate concentration at the low supplementation doses of BC, no inhibiting effect of dose of BC occurred for CH_4 formation. However, as CH_4 formation was estimated from the concentrations of VFA produced during *in vitro* fermentation (Moss et al., 2000), results have to be interpreted with caution. In general, ruminal CH_4 formation is negatively correlated with an increase in the propionate proportion, and a decline in the acetate:propionate ratio (Russell, 1998). In the rumen, propionate formation competes with CH_4 for hydrogen, while acetate and butyrate are considered to promote CH_4 production (Moss et al., 2000).

Because effects of some BC may be pH and diet dependent, and their use may be beneficial only under specific conditions and production systems (Calsamiglia et al., 2007), we examined the influence of diet composition on BC efficiency using the backward elimination procedure. This analysis, by adding the chemical composition of the diet together with BC, improved the accuracy of estimation of several fermentation variables. In doing so, we can confirm that inclusion of the chemical composition of the incubated diet into the same model with BC supplementation affected the predicted responses of several variables. Therefore, optimisation of the efficacy of BC on ruminal microbial activity must involve proper diet formulation to prevent diet composition rendering BC ineffective. Our findings suggest that effects of BC on microbial growth must be seen in close association of substrate related shifts in bacterial composition and activity. The strongest relation between BC effect and diet ingredients occurred for butyrate proportion, followed by relations for total VFA concentration and IVDMD and IVNDFD, whereas effects of BC on acetate and propionate proportion and CH_4 and ammonia formation were less well related to dietary NDF and CP. For instance, the results indicate that dietary NDF potentiated the effect of BC on total VFA, whereas it counteracted effects of BC on acetate and propionate proportion and acetate:propionate ratio, ammonia, IVDMD, IVNDFD and CH_4 formation. Dietary NDF is a structurally complex substrate which is generally more slowly fermented than less complex carbohydrates such as starch (Tafaj et al., 2007; Penner et al., 2009; Martínez et al., 2010) leading to an overall lower production of VFA thereby favouring acetate and CH_4 production. In contrast, dietary CP content potentiated the effect of BC on total VFA concentrations, IVDMD and IVNDFD, whereas it counteracted effects of BC on molar proportions of propionate and butyrate, acetate:propionate ratio and ammonia release. Increasing dietary CP content likely reduced the carbohydrate (*i.e.*, fibre and starch) proportion of the diet thereby reducing available substrate for propionate and butyrate formation. Indeed, propionate and butyrate are generated during microbial degradation of carbohydrates, such as plant polysaccharides, oligosaccharides and sugars (Louis et al., 2007). With increasing dietary CP content, ammonia in ruminal fluid increased. Therefore, increasing substrate availability for microbial protein degradation may have lessened the effect of BC on ammonia formation. An optimal balance of CP and carbohydrates is required for optimal rumen bacterial growth and nutrient degradation *in vitro* (Hoover and Stokes, 1991). Results of the backward elimination analysis for *in vitro* fermentation patterns emphasize that poorer synchronization of protein and carbohydrate availability may interfere in achieving desirable effects of dietary supplementation of BC.

5. Conclusions

Overall, our evaluation indicates that supplementation dose of ruminally bioactive compounds (BC) and the chemical composition of the incubated diet are factors determining responses of BC on ruminal fermentation and degradation. However, as BC are highly variable and differ in efficiency, responses also depend on BC composition, the results from our study do not apply to all BC. Nevertheless, the findings may have general implications for *in vivo* conditions, and more *in vivo* research is warranted to validate our findings and to determine optimal contents of CP and NDF, in particular, in the diet to obtain optimal effects of BC on ruminal fermentation.

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