Short communication: Sodium salicylate negatively affects rumen fermentation in vitro and in situ

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ABSTRACT

Administration of sodium salicylate (SS) to cows in early lactation has a positive effect on whole-lactation milk production but a negative effect on metabolism in some cases. The objective of this trial was to determine whether SS directly affects rumen fermentation. Experiment 1 was designed to investigate the effects of direct inclusion of SS in a 24-h batch culture, and experiment 2 was designed to test the fermentative ability of rumen fluid from heifers who had received SS. In experiment 1, we combined strained and pooled rumen fluid from 3 heifers in a 2:1 ratio with McDougall's buffer, and added 150 mL of the inoculum to each flask ($n = 5/\text{treatment}$) with 2.5 g of fermentation substrate similar to a lactating cow ration, ground to 1 mm. We then added premixed treatments (1-mL volume) to achieve the desired final amount of SS (CON1 = 0 mg, LOW = 125 mg, MED = 250 mg, HI = 375 mg). In experiment 2, 6 heifers ($n = 3/\text{treatment}$) were drenched daily for 3 d, either with 62.5 g of SS dissolved in water (SAL) or an equal volume of water (CON2). Rumen fluid was collected from each heifer and was not pooled. After the fluid was mixed 2:1 with McDougall's buffer, 150 mL of inoculum was added to the fermentation flasks ($n = 4/\text{heifer}$) with 2.5 g of fermentation substrate. This experiment was performed the day before SS treatment began and repeated 1, 13, and 35 d after the end of the treatment period. We also performed an in situ experiment at each of these time points. In the first experiment, inclusion of SS resulted in a decrease in dry matter disappearance (DMD) over 24 h, as well as an increase in final pH. We detected no difference between treatments for gas production asymptotic volume, rate, or lag. In the second experiment, we detected a significant treatment $\times$ day interaction for DMD: we observed no difference between groups during a 24-h

batch culture on the day following treatment, but SAL resulted in lower DMD on d 13 and d 35. We detected no treatment effect on the final pH of the batch culture or on any gas-production parameters. We observed a tendency for SAL to decrease the DMD rate in situ on the day after treatment. These results indicate that SS administration has a negative effect on rumen microorganisms.

Key words: rumen fermentation, sodium salicylate, in vitro

Short Communication

During transition into lactation after calving, dairy cattle experience elevated systemic inflammation. Administration of the anti-inflammatory medication sodium salicylate (SS) after calving has been shown to increase whole-lactation milk production in multiparous dairy cows, but treatment with SS has been associated with hypoglycemia in some circumstances. Farney et al. (2013a) reported that cows had decreased blood glucose concentrations after receiving SS in their drinking water for 7 d after calving, but 305-d milk yield was greater in older cows (parity 3 and greater) that received SS compared with controls (Farney et al., 2013b). In a follow-up study, Carpenter et al. (2016b) demonstrated that giving 3 daily doses of SS after calving in multiparous cows did not result in the same hypoglycemia, but 305-d milk yield was still increased.

Salicylic acid functions as a hormone in plants to combat pathogens. It has anti-inflammatory properties in mammals via its interactions with the nuclear factor (NF)- κ B pathway, which is involved with gene expression during inflammation and infection (Kopp and Ghosh, 1994). Besides SS, other forms of salicylate have also been shown to be antimicrobial, with the ability to depress rumen microbial function. Ruiz-Moreno et al. (2015) administered bismuth subsalicylate (BSS) to rumen microbes in a batch culture in an attempt to reduce hydrogen sulfide production resulting from the fermentation of distillers grains. When BSS was included at 2 and 4% of DM, final pH was increased, and total VFA concentration was decreased, at 4% of

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Table 1. Ingredient and chemical composition of fermentation substrate for in vitro rumen fermentation experiments

Item	% of DM
Corn silage	22.0
Alfalfa hay	21.0
Corn grain	25.0
Cottonseed	4.0
Dried distillers grains	14.0
Soybean meal	14.0
CP	21.5
NDF	24.8
ADF	18.9
Ether extract	3.9

DM. When BSS was added during continuous culture fermentation at 1% of DM, total VFA concentrations were also decreased, and pH and digestion of OM, NDF, and ADF were increased. Similarly, Fessenden (2013) reported that when BSS was administered at 0 or 0.5% of diet DM (at 2 different levels of sulfur), total VFA concentrations were decreased and pH was increased, and OM digestion was also decreased.

Experiments with human colonic bacteria have also shown an antimicrobial effect of BSS. In the stomach, BSS is hydrolyzed to form salicylic acid (Bierer, 1990). Salicylate is believed to be partially responsible for the antibacterial effects of BSS. Cornick et al. (1990) reported that although SS was as active as BSS against aerobic bacteria, it was not as active against anaerobic bacteria, such as those found in the rumen, although inhibitory activity was still observed. León-Barúa et al. (1990) demonstrated that BSS reduced gas production by colonic bacteria in vitro to a greater extent than other bismuth-containing compounds. Additionally, Manhart (1990) showed a dose-dependent inhibition of various strains of bacteria to SS.

The objective of these experiments was to determine the effect of SS on in vitro fermentation by rumen microorganisms. In the first experiment, SS was directly included in a dose-dependent manner in batch cultures of rumen bacteria. In the second experiment, SS was administered to heifers, and the rumen fluid collected from these animals was tested for its fermentative ability in batch culture.

In experiment 1, SS was added directly to batch cultures of rumen fluid at different amounts (**CON1** = 0 mg, **LOW** = 125 mg, **MED** = 250 mg, **HI** = 375 mg). Rumen fluid was collected from 3 heifers, strained through 8 layers of cheesecloth immediately following collection and 4 layers of cheesecloth immediately before flocculation, and allowed to incubate in the laboratory for approximately 1 h to allow it to stratify. The bottom layer was discarded, and the middle layer was collected for pooling. Pooled fluid was combined

in a 2:1 ratio with McDougall's buffer, and 150 mL of the inoculum was added to each 250-mL flask ($n = 5/\text{treatment}$). Five blank flasks contained inoculum alone, and each treatment and control flask contained 2.5 g of fermentation substrate (Table 1). Before inoculum was added to the flasks, 1 mL of premixed treatment was added to achieve the desired final amount of SS. Cumulative gas pressure was measured using the Ankom^{RF} Gas Production System (Ankom Technology, Macedon, NY). Vessel pressure was recorded at 5 min intervals. Vessels were incubated at 39.5°C for 24 h. Following incubation, fermentation was inhibited by placing fermentation vessels on ice. At this time, final pH was measured and fermentation vessels were emptied into individual containers and dried at 60°C for 48 h. Dry matter disappearance (**DMD**) was quantified as the proportion of dry matter remaining, less the approximate contribution of DM from the rumen fluid (estimated by final dry weight of the content of the blank flasks) as a proportion of the initial DM of the substrate.

In experiment 2, 6 heifers [age = 10 mo, standard deviation (**SD**) = 0.3; BW = 614.2 kg, SD = 44.87; $n = 3/\text{treatment}$] were drenched daily for 3 d with either 62.5 g of SS in water (**SAL**) or an equal volume of water (**CON2**) approximately 12 h after feeding. Each heifer received the same high-forage ration (CP = 11.9% of DM; NDF = 48.5% of DM; ADF = 32.3% of DM; ether extract = 2.5% of DM). Four batch cultures were performed as described above with the exception that rumen fluid was not pooled and SS was not added to the inoculum, such that heifer was the experimental unit. Rumen fluid was collected approximately 14 h after the final dose of SS. Inoculum from each heifer was replicated in 4 flasks with 2.5 g substrate added, and 2 flasks without substrate functioned as blanks for each heifer. Batch cultures were performed on the day before the start of treatment, as well as at 1, 13, and 35 d following treatment. During each batch culture, heifers were handled in blocks containing 1 CON2 and 1 SAL animal, in an attempt to prevent bias between treatments due to handling.

An in situ experiment was performed in parallel with experiment 2 to estimate the rate of substrate degradation in the rumen. Immediately following each rumen fluid collection, we inserted 2 Dacron bags (5 × 10 cm; 50 µm porosity) containing approximately 1 g of substrate DM (Table 1) into the rumen of each heifer for each time point. Time points before removal of the bags from the rumen were 2, 8, 16, 24, and 48 h. Additionally, 12 bags were rinsed under running water and washed with the other bags upon removal from the rumen to estimate solubility. After rinsing or diges-

tion, bags were dried in a forced-air oven at 105°C and weighed to measure the remaining dry matter.

In both experiments, we estimated gas variables— asymptotic volume, lag, and rate—using the NLIN procedure of SAS 9.3 (SAS Institute Inc., Cary, NC), which obtains estimates using nonlinear least squares. We used the following model to obtain estimates:

$$x = \frac{v}{1 + e^{[2-4k(t-l)]}},$$

where v = total volume of gas produced (mL), k = rate of gas production (mL/min), t = time (min), and l = lag in gas production (min). After obtaining estimates for each flask, gas-production variables were analyzed for each experiment as described for other variables.

Data from experiment 1 were analyzed using the GLM procedure of SAS, with treatment as the independent variable and gas production metrics, DMD, final pH, and the change in pH as dependent variables. Data from experiment 2 were analyzed using the MIXED procedure of SAS. Each dependent variable was analyzed with its own value on the first day of the experiment (baseline) as a covariate. Besides these covariates, the model included the fixed effects of treatment, day, and treatment $\times$ day interaction. The random statement contained heifer and replicate within heifer, and repeated measures were used across days within heifer. An autoregressive(1) covariance structure was selected based on Bayesian information criterion value.

In experiment 1, DMD was depressed by the inclusion of SS ($P < 0.05$): HI had lesser DMD than LOW ($P < 0.05$), and MED was intermediate (Table 2). Final pH was similar for LOW and CON1, but MED and HI had a higher final pH than CON1 ($P < 0.05$; Table 2). We observed no differences in gas production for asymptotic volume, rate, or lag ($P \geq 0.28$; Table 2).

The results for experiment 2 are reported in Table 3. We observed no influence of SAL on the final pH of batch cultures at any time point ($P = 0.71$). The final pH of the batch culture performed before treatment

administration was a significant predictor of final pH at all time points after treatment ($P = 0.03$). Immediately after treatment, SAL had no effect on DMD ($P = 0.67$); however, treatment reduced ($P < 0.01$) DMD in batch culture 13 d after treatment, and DMD was still reduced ($P = 0.01$) 35 d after SAL treatment (treatment $\times$ day: $P = 0.01$). For the in situ portion of experiment 2, we detected no differences due to treatment for rate of DMD ($P = 0.21$). We observed a significant effect of treatment and day (both $P \leq 0.01$) on DMD at 48 h (treatment $\times$ day: $P = 0.31$). Based on these results, it would appear that SS treatment causes long-term inhibition of rumen fermentation or digestion.

As discussed above, other salicylate compounds—specifically BSS—have negative effects on rumen fermentation. Our findings are similar to those of Ruiz-Moreno et al. (2015), who showed that BSS inclusion at 2 and 4% of DM in batch cultures increased final pH at 24 h. In continuous culture, inclusion of BSS at 1% of DM increased average pH, although digestion of OM was also increased (Ruiz-Moreno et al., 2015). Similar to the current study, however, Fessenden (2013) reported that inclusion of BSS at 0.5% of DM in continuous culture decreased true and apparent DM and OM digestion, with a corresponding increase in average pH. For comparison, the present experiment included SS at approximately 5, 9, and 13% of substrate DM for LOW, MED, and HI, respectively. It should be noted, however, that BSS is a larger molecule than SS, at a molecular weight of 362.09 g/mol, compared with 160.10 g/mol for SS. Salicylic acid itself has a molecular weight of 138.12 g/mol. Therefore, the salicylate component of BSS is roughly 38% of its molecular mass, but it composes approximately 85% of the SS molecule. Based on these calculations, BSS at 2 or 4% of DM is approximately equivalent to salicylate inclusion at 0.8 or 1.5% of DM, respectively, but SS levels at 5, 9, and 13% of DM is approximately equivalent to salicylate inclusion at 4, 8, and 11% of DM, respectively. If in vitro substrate DM is considered to be roughly the equivalent of DMI, this level of inclusion would be much higher than recommended in vivo. However, this assumes that the ratio

Table 2. Effects of adding sodium salicylate¹ at different amounts on fermentation by rumen microbes in vitro for 24 h (experiment 1)

Item	CON1	LOW	MED	HI	Pooled SEM
Final pH	6.31 ^a	6.36 ^{ab}	6.42 ^b	6.45 ^b	0.01
24-h disappearance (% of DM)	48.8 ^a	37.08 ^b	29.59 ^{bc}	22.78 ^c	1.96
Gas-production measurements					
Asymptotic volume (mL)	249.8	297.9	276.2	296.0	26.41
Rate (mL/min)	0.0010	0.0010	0.0008	0.0010	0.0002
Lag (min)	137.0	122.2	79.1	127.8	55.09

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹Sodium salicylate was added to rumen fluid inoculum at the beginning of a 24-h batch culture of mixed rumen microbes in buffer; CON1 = 0 mg of sodium salicylate, LOW = 125 mg, MED = 250 mg, HI = 375 mg; $n = 5$ flasks/treatment.

Table 3. Effects of drenching heifers with sodium salicylate¹ on the fermentation capacity of rumen microbes in vitro for 24 h and in situ (experiment 2)

Item	CON2			SAL			Pooled SEM
	d 1	d 12	d 35	d 1	d 12	d 35	
Final pH	6.34	6.34	6.25	6.32	6.34	6.29	0.018
24-h disappearance ² (% of DM)	46.5 ^a	44.9 ^a	43.9 ^a	46.0 ^a	38.9 ^b	40.3 ^b	0.93
Gas production							
Asymptotic volume (mL)	317.1	365.6	276.3	311.3	364.3	281.2	3.69
Rate (mL/min)	0.0011	0.0012	0.0015	0.0010	0.0012	0.0016	0.0003
Lag (min)	87.5	180.7	125.6	97.1	168.7	124.0	8.18
In situ measurements							
DM disappearance rate (%·h ⁻¹)	11.6	12.0	11.7	8.6	9.6	10.7	1.18
48-h disappearance ³ (% of initial DM)	91.3	91.2	92.0	90.4	90.8	91.7	0.33

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹Heifers were drenched with 62.5 g of sodium salicylate (SAL) or water (CON2) for 3 d, and in vitro and in situ experiments were conducted at 1, 12, and 35 d after treatment administration to test the ability of rumen microorganisms to ferment substrate.

²Means differed due to treatment ($P < 0.01$), day ($P < 0.01$), and the interaction between treatment and day ($P = 0.01$).

³Means differed due to treatment ($P = 0.01$) and day ($P < 0.01$; treatment $\times$ day: $P = 0.31$).

of substrate to volume in vitro is roughly equivalent to the ratio of digesta to volume in the rumen, which is not the case. Alternatively, the ratio of SS to volume in the flasks for MED was roughly equivalent to 125 g of SS in a rumen volume of 65 L, meaning that the SS concentration in the solution was at approximately physiological levels for this treatment. Regardless, the results of experiment 2 indicate an effect of SS administration on the ruminal environment at physiologically relevant levels.

Unlike the results presented here, Ruiz-Moreno et al. (2015) reported a significant decrease in gas production during 24-h batch culture with increasing levels of BSS inclusion. This was likely due to differences in measurement of gas production. Ruiz-Moreno et al. (2015) measured gas production by displacement of water when batch cultures were performed in serum bottles; the present study used the Ankom^{RF} system. We hypothesize that the simplistic measurement used by Ruiz-Moreno et al. (2015) reduced measurement variation compared with the present experiment, increasing the statistical power of gas-production measurement.

These results appear to be counterintuitive to previous accounts that showed an increase in milk production in response to SS treatment in early lactation, but they may help to explain the metabolic outcomes observed in these studies. Despite the positive effects on milk production observed in older cows, cows in their second and higher parity experienced hypoglycemia in early lactation under certain experimental conditions (Farnley et al., 2013a). This coincided with a higher value in the revised quantitative insulin sensitivity check (RQUICKI; an estimate of relative insulin sensitivity (Holtenius and Holtenius, 2007) on d 7 of treatment

without any differences in expression of rate-determining hepatic gluconeogenic enzymes. It is feasible that this observation was a result of decreased production of glucogenic precursors in the rumen, possibly in addition to the effects of SS on insulin sensitivity. In fact, decreased substrate availability in the liver may have served to enhance insulin sensitivity in these animals. Bjerre-Harpøth et al. (2012) reported that cows in early lactation who underwent feed restriction experienced a significant change in RQUICKI values and had a higher ratio of glucagon to insulin, potentially indicating enhanced insulin sensitivity. Lower amounts of propionate from ruminal fermentation due to depression in microbial activity or changes in bacterial community composition could have the same effect.

Precedents can be found in the literature for sustained effects after modification of the microbial population, but to our knowledge, this experiment was unique in the length of time we observed a difference after treatment administration. Weimer et al. (2010b) noted that bacterial community composition did not completely return to its original state up to 4 wk after monensin was removed in combination with a milk-fat-inducing diet. Although exceptions such as this exist, it is difficult to force shifts in the rumen microbial population for an extended period of time (Weimer, 1998). Even after almost complete exchange of rumen contents, microbial populations in the rumen demonstrate a specificity for the host that is difficult to overcome by non-host forces (Weimer et al., 2010a). This is why experiments using a Latin square design to study rumen modifiers can successfully implement a wash-out period to minimize carry-over effects. Considering this information, the results of the current experiment are surprising.

Other research has shown long-term alterations in feeding behavior in response to SS administration in lactating cows (Carpenter et al., 2016a). It is likely that these observations and the findings of the present experiments are related, but further research is needed to determine the cause-and-effect relationship between ruminal fermentation and feeding behavior after SS treatment. Some programming effect of the rumen microbial population may have resulted in a long-term shift that changed the fermentative ability of the rumen, but forces outside of the rumen (such as a neurological effect) could also have altered feeding behavior, in turn changing the rumen environment and resulting in a population shift. Unfortunately, we did not collect data on DMI and feeding behavior for the heifers used in experiment 2.

Although these experiments were relatively simple, they strongly suggest an antimicrobial effect of SS on rumen microorganisms. We observed not only an immediate and dose-dependent effect of this compound *in vitro*, but also a sustained negative effect on the ability of rumen microorganisms to degrade DM. Several questions remain. We did not perform an analysis on VFA production or profile *in vitro* or *in vivo*, nor did we characterize microbial populations following SAL in experiment 2. Future research should focus on these questions, as well as the effects on the rumen *in vivo*. It is unclear why a sustained positive response in milk production has been observed following SS administration in early lactation when the evidence here indicates that rumen function is hampered. Future research to explore this relationship, as well as the relationship between SS and feeding behavior, is clearly warranted.

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